Characterization of Staphylococci and Evaluation of Methods for Detection of Methicillin Resistant *Staphylococcus aureus*

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CERTIFICATE

This is to certify that this dissertation work entitled **"Characterization of Staphylococci and Evaluation of Methods for Detection of Methicillin Resistant *Staphylococcus aureus*"** is a bonafide record of work done by **Dr.Anila.A.Mathews**, in the **DEPARTMENT OF MICROBIOLOGY**, P.S.G. Institute of Medical Sciences & Research, Coimbatore – 641 004, under the effective guidance of Dr Marina Thomas, **M.D.,** during the period of study (2005-2008).

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INTRODUCTION

Staphylococci are Gram-positive cocci that belong to the family Micrococcaceae. They are the commonest of all clinical isolates and are responsible for several suppurative types of infections. They have a differential ability to spread and cause outbreaks in hospitals.[1] However, due to development of methicillin resistance in *Staphylococcus aureus* isolates; treatment of these infections has become problematic. Indiscriminate use of multiple antibiotics, prolonged hospital stay, intravenous drug use, carriage of Methicillin resistant *Staphylococcus aureus* (MRSA) in nose are important risk factors for MRSA acquistion[2]

Staphylococcus aureus produces a series of enzymes and toxins [5] and is a major pathogen causing skin abscess, wound infections, osteomyelitis, endocarditis, pneumonia, meningitis, bacteremia and toxic shock syndrome.[3] Originally, Penicillin was the drug of choice for treatment of serious *Staphylococcus aureus* infections. The emergence of its resistance to penicillin was due to acquisition of a plasmid borne genetic element coding for beta lactamase production. Semi synthetic, penicillinase resistant penicillins such as oxacillin and methicillin then became the drug of choice. Gradually, over the years, *Staphylococcus aureus* has evolved resistance to many antibiotics especially methicillin. Methicillin resistance first appeared among nosocomial isolates of *Staphylococcus aureus* in 1961[6]and resistance is due to the presence of altered penicillin –binding protein called PBP2a that results from acquisition of a chromosomal gene called mecA. The incidence of methicillin resistant *Staphylococcus aureus* (MRSA) in India ranges from 30 to 70%.[8] Since MRSA's are resistant to all β- lactam antibiotics, the therapeutic options are limited significantly. Moreover, the incidence of nosocomial infections caused by MRSA continues to increase world wide, and therefore the importance of their detection, especially for therapeutic and epidemiological purposes arises. Hence methods used to detect MRSA in clinical samples should have high sensitivity and specificity and most importantly the result should be available within a short time. There are many phenotypic methods available for detection of MRSA. These

include Oxacillin and Cefoxitin resistance detection by disc diffusion method, Oxacillin resistance agar screening, MIC of > 4µg/ml to oxacillin, and Detection of PB2a by latex agglutination etc. Most laboratories detect MRSA by testing its susceptibility to Oxacillin 1µg by the Kirby Bauer disc diffusion technique. Problems in detection of MRSA have been reported with all the above methods, mainly due to the variability seen in the standard of techniques. Therefore, the Gold standard as of now, for the determination of MRSA, is by detection of MecA and Fem A gene by PCR. Traditionally MRSA has been considered as major nosocomial pathogen in health care facilities, but in the last decade it has been observed to be emerging in the community as well. The emergence of a community pathogen depends on its ability to survive in different environments and to interact successfully with the host. *Staphylococcus aureus* is one of the most successful and adaptable human pathogens.[10] The community acquired strains tend to be more susceptible to non beta lactam agents as compared to hospital acquired MRSA isolates and appear to carry a unique staphylococcal chromosome (SCC mec type IV) in relation to resistant gene.[7] MRSA's are highly virulent strains capable of clonal dissemination and have the ability to cause epidemics of furunculosis and other skin and soft tissue infections, irrespective of characteristics of populations or the setting. The community onset MRSA carrying the SCC mec type IV element possesses a serious threat and is likely to emerge as a major public health concern. The emergence of MRSA infections in the community places renewed emphasis on the importance of non-antibiotic management of localized infections. Although sometimes neglected, appropriate drainage is the definitive management of many skin and soft tissue infections and is always an important adjunct to antibiotic therapy in deeper, closed space infections.

Coagulase negative staphylococci have long been regarded as harmless skin commensals and therefore were dismissed as contaminants. Their role as pathogens has been recognized only recently. The increasing incidence of infections caused by this bacteria can be attributed to their particular affinity for foreign materials such as prosthetic valves, devices and intravascular catheters.[9] Use of such invasive technologies in patients who are sick, immunocompromised and at the extremes of age, has brought Coagulase negative Staphylococci to the forefront of nosocomial pathogens, resulting in considerable morbidity and spiraling medical

cost. Among the staphylococci, the two coagulase negative species(CONS), Staphylococcus *epidermidis* and *Staphylococcus saprophyticus*, are seen frequently in human infections.[3] Other staphylococci like *Staphylococcus haemolyticus*, *Staphylococcus hominis, Staphylococcus simulans, Staphylococcus cohini* etc although are found on human skin are sometimes implicated as the causative organisms in septicemia, osteomyelitis, catheter related sepsis and native valve endocarditis.[4, 9]

Methicillin resistant Coagulase negative staphylococci (MRCONS) have also emerged in hospitalized patients and patients who have undergone prosthetic heart valve surgeries. Most infections due to CONS are nosocomial and it is not surprising therefore, that these infections have become increasingly resistant to multiple antibiotics. About 80-90% of CONS isolated from human specimens produce an inducible beta lactamase. Further- more 60-80% of nosocomial CONS are methicillin resistant. Methicillin resistant strains are often resistant to macrolides, lincosamides, aminoglycosides, fluroquinolones and other antimicrobial agents and emergence of strains with reduced susceptibility to glycopeptides has raised concern about development of strains resistant to all antibiotics.

Therefore recognition of isolates of *Staphylococcus aureus* as MRSA continues to be a task that clinical microbiologist should pursue diligently and report as accurately and as rapidly as possible. Renewed emphasis on the prevention of MRSA infections is also necessary. Control of MRSA in the health care setting remains an important means of limiting its spread in the community.

This study aims at characterizing all Staphylococci including clinically relevant Coagulase negative staphylococci and comparison of various methods used for detection of methicillin resistance and also includes the comparison of the antibiotic susceptibility pattern of CAMRSA v HAMRSA.

AIMS AND OBJECTIVES

1. To isolate and identify Staphylococcal species from different clinical samples at PSG Hospitals, Coimbatore.
2. To determine the incidence of Methicillin Resistant *Staphylococcus aureus* (MRSA) and compare between the phenotypic and genotypic methods for detection of MRSA.
3. To compare the antibiotic susceptibility pattern between MSSA and MRSA, community acquired MRSA & hospital acquired MRSA and also to analyse the susceptibility pattern of Coagulase Negative Staphylococci

REVIEW OF LITERATURE

Staphylococci are gram-positive cocci belonging to family Micrococacceae. The term Micrococci is applied to a large variety of gram positive and mostly catalase positive cocci, some of which are saprophytes, widely distributed in nature, whereas others are parasitic and potentially pathogenic. They occur in pairs, small clusters and tetrads. The two genera Staphylococcus and Micrococcus are generally recognized and separated on the ability of staphylococcus to grow and produce acid from glucose anaerobically and its susceptibility to lysis by lysostaphin. Micrococcocci is negative for both the test. The international Sub committee on taxonomy of Staphylococci and micrococci has recommended adoption of standard method for distinguishing these organisms by anaerobic utilization of carbohydrates. Accordingly, if the standard test is used, those organisms which utilize glucose fermentatively (anaerobically) and are lysed by 1μ/ml lysostaphin endopeptidase are designated as Staphylococcus and those that do not produce acid from glucose or do so oxidatively (aerobically) only are designated as Micrococcus. Bergy's manual includes additionally the genus Planococcus in the Micrococcaceae and the genera Aerococcus, Pediococcus and Leuconostoc in the family Streptococcaceae where as the International subcommittee on the nomenclature of staphylococci and Micrococci places Aerococcus in the Micrococcaceae. Planococcus and Leuconostoc do not cause human infections.[11]

History

In 1871,Von Reckling Hausen first observed Staphylococci in pus. It was cultivated on liquid media by Louis Pasteur (1880) and was shown by Alexander Ogston, a Scottish surgeon in the year 1881, to be frequently associated with acute and chronic diseases. He also gave it the name *Staphylococcu*s. Ogston also noticed that non- virulent Staphylococci were also present on the skin surface. Rossenbach in 1884 made a detailed study, obtained them in pure culture and divided them into two species *Staphylococcus aureus* and *Staphylococcus albus*

Passet in the year 1885 added another species *Staphylococcus citreus*. Welch later named *staphylococcus albus* as *Staphylococcus epidermidis* in the year 1891. This distinction was of considerable importance, since *Staphylococcus*

aureus was a major cause of mortality and morbidity and clinical specimens often carried both type of organisms. Von daranyi in year 1925 was the first person to draw attention to the partial value of the coagulase test to identify *Staphylococcus aureus*, and it still remains the most important test used in clinical laboratory to identify this species. Until 1975 Coagulase Negative Staphylococci were grouped together as *Staphylococcus epidermidis*, distinguished by their inability to clot blood plasma. In 1975, Kloos Schleiferi extended the existing identification scheme by adding seven new species to the already known *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. Today there are about 32 species of CONS and about 15 of them are indigenous to man while the remaining are non-human pathogens. During the past few decades, importance of CONS as a human pathogen is being recognized. In year 1994-1995, the national nosocomial infection surveillance program reported that CONS were the causative agent in 11% of all nosocomial infection, making this pathogen the third most common nosocomial isolate. CONS equal or surpass *Staphylococcus aureus* as a cause of device related nosocomial infection. *Staphylococcus epidermidis* is a common member of the normal flora of skin and mucous membranes. Its large numbers and ubiquitous distribution make it one of the most commonly isolated organisms in the clinical laboratory. While at one time the appearance of *Staphylococcus epidermidis* in clinical material could be dismissed as contamination, it is now one of the most important agents of hospital acquired infections. Coagulase negative staphylococci, notably Staphylococcus epidermidis may produce slime which envelops cells and aids their adherence to and accumulation on the surfaces of medical devices such as catheters and prosthesis.[3]Immunosuppressed or neutropenic patients are particularly at risk, as are individuals with indwelling catheters or prosthetic devices. It can also cause endocarditis in individuals with previous heart valve damage. The hydrophobic nature of the organism's cell surface facilitates its adherence to synthetic devices as well as damaged heart valves. Following initial colonization, a copious amount of extra cellular polysaccharide or slime is synthesized, forming a protective biofilm around the colony. Because many isolates are multiple antibiotic resistant, these infections are very serious and can even be fatal.

In a series of clinical observations and laboratory studies published in 1880 and 1882, Ogston described staphylococcal disease and its role in sepsis and abscess formation. More than 100 years later, *Staphylococcus aureus* still remains a versatile and dangerous pathogen in man. The frequencies of both community-acquired and hospital-acquired staphylococcal infections have increased steadily, with little change in overall mortality. Treatment of these infections has become more difficult because of the emergence of multi drug-resistant strains.

Morphology

Staphylococci are spherical organisms with a diameter of 0.8-1μm in size. In films of pus or solid media, they appear as grape like cluster with a few single and paired Cocci, whereas in broths they appear as small groups, pairs, singles and short chains (less than five cocci in line). They are Gram Positive, nonsporing, nonmotile and except for strains, which have a microcapsule, most are noncapsulate[3, 4,5,11]. Of the 11 types of micro capsular polysaccharide serotypes that have been identified, types 5 and 8 account for 75 percent of human infections. Most methicillin-resistant *Staphylococcus aureus* isolates are type 5. The chemical composition of four of these antiphagocytic polysaccharides, including types 5 and 8, has been determined, and all four have been shown to be chemically related. [16] Differences in the peptidoglycan structure of staphylococcal strains may contribute to variations in their capacity to cause disseminated intravascular coagulation. Peptidoglycan may have endotoxin-like activity, stimulating the release of cytokines by macrophages, activation of complement, and aggregation of platelets. Ribitol teichoic acids, covalently bound to peptidoglycan, are major constituents of the cell wall. Lipoteichoic acid is a glycerol phosphate polymer linked to the glycolipid terminus anchored in the cytoplasmic membrane. [16]

Surface Proteins

A ligand-binding domain at the N terminal of secretory signal sequence that is exposed on the surface of the bacterial cell enables some of these proteins to function as adhesins. Protein A, the prototype of these proteins, has antiphagocytic properties that are based on its ability to bind to the Fc portion of immunoglobulins.

Several of these related proteins bind extra cellular-matrix molecules and have been designated microbial-surface components recognizing adhesive matrix molecules (MSCRAMM). Recent studies suggest that these proteins play an important part in the ability of staphylococci to colonize host tissue.

Cultural Characteristics

Staphylococci grow well on blood free basal media. They are facultative anaerobes having an optimum ph between 7.4-7.6 and temperature between 35-37ºC.They are also able to grow between 12-44 ºC. After aerobic incubation on nutrient agar for 24 hours, the colonies are 2-3mm in diameter, have a smooth glistening surface, entire edge, butyrous consistency and opaque, pigmented appearance. Colonies are smaller and pigmentation is absent on plates incubated anaerobically .[3,4,5,11]

Pigmentation

Golden (orange, yellow and cream to buff varieties), white or may be poorly developed in 24 hours. It is enhanced by prolongation of incubation for 48 hours or incubation at room temperature or by use of pigment enhancing medium such as milk agar (33%full fresh milk), cream agar (10% cream) or glycerol monoacetate (1%) spreading a colony on white filter paper and allowing it to dry is simple and satisfactory a way of detecting pigment .The yellow pigments which are triterpenoid carotenoids are located in the cellmembrane[3.] Many strains are beta hemolytic on blood agar. Sheep's blood is recommended because sheep's RBC are lysed by alpha toxin which is considered to be most important staphylococcal toxin, whereas human RBC are lysed by delta toxin. Macconkey agar-Colonies are pinkish (lactose fermenting) and very small to normal in size depending on the batch of the medium Broths: Uniform turbidity is seen with some powdery deposit.[3,4,5]

Morphology variants

Prolonged incubation particularly important to detect morphology variants such as small colony variants (SCV). SCV's grow into tiny colonies that are difficult to distinguish. They result from mutations in the respiratory chain and possibly other kinds of mutations that are yet to be known. SCV's are more tolerant to killing by a number of

additional antibiotics like aminoglycosides, beta lactams and glycopeptides. SCV's are infective as or more than other fast growing ones.[3, 11]

Selective media[11]

Salt Media

a) Columbia agar with added salt

b) Muller Hinton agar with 2% salt

c) Mannitol salt agar

This medium is used in addition to blood agar when contamination with other bacteria is expected because high content of sodium chloride (7.5%) and presence of mannitol and phenol red in this medium, *Staphylococcus aureus* will appears yellow colony surrounded by yellow zone where as other Staphylococci usually form a small colony in red or purple zone and other organisms including gram negative organisms are usually completely inhibited. So manitol salt agar is an indicator medium also

d) Staphylococcus medium no110 and Chapman stone agar

These high salt content media containing mannitol and gelatin are now used less frequently than before since gelatin liquefaction is no longer considered a differential test in classification.

e) Tojel Johnson medium supplemented with 0.5% pyruvate and salt milk agar

Tellurite containing media

a) Baird Parker agar containing potassium tellurite and lithium chloride as selective agents.

b) Tellurite glycine agar: It is a medium containing glycine, lithium chloride and potassium tellurite as selective agents. Coagulase positive Staphylococci produce black colonies where as Coagulase negative Staphylococci or other organisms fail to produce visible growth in 24 hrs or form grey colonies.

Mannitol Neomycin agar

This consist of trypticase soy agar (1 lr) mannitol (10g) Neomycin sulphite (0.0005g) phenol red (0.0025g) and defribrinated sheep's blood (50 ml). This medium is

used for isolation and identification of Staphylococci. This medium inhibits Coagulase Negative Staphylococci and micrococci but not Coagulase positive Staphylococci, Streptococcus, Proteus, Pseudomonas and Candida.

Indicator media[3,4,11]

Phenophathalein diphosphate agar

This indicator media allows provisional identification of colonies of *Staphylococcus aureus* in mixed cultures. Few colonies of CONS like *Staphylococcus epidermidis* are also phosphatase positive.

Toxins and Enzymes

Staphylococci produce numerous toxins that are grouped on the basis of their mechanisms of action. Cytotoxin, such as the 33-kd protein-alpha toxin, cause pore formation and induce proinflammatory changes in mammalian cells. The consequent cellular damage may contribute to manifestations of the sepsis syndrome. The pyrogenic-toxin super antigens are structurally related, sharing various degrees of amino acid sequence homology. They function as super antigens by binding to major Histocompatability complex (MHC) class II proteins, causing extensive T-cell proliferation and cytokine release. Different domains of the enterotoxin molecule are responsible for the two diseases caused by these proteins, the toxic shock syndrome and food poisoning. Despite little amino acid sequence homology, toxic shock syndrome toxin 1 is structurally similar to enterotoxin B and C. The gene for toxic shock syndrome toxin 1 is found in 20 percent of *Staphylococcus aureus* isolates. The exfoliate toxins, including epidermolytic toxins A and B, causes skin erythema and separation, as is seen in the staphylococcal scalded skin syndrome. The mechanism of action of these toxins remains controversial. Panton–Valentine leukocidin is a leukocytolytic toxin that has been epidemiological associated with severe cutaneous infections. Staphylococci produce various enzymes such as protease, lipase and hyaluronidase that destroy tissue. These bacterial products may facilitate the spread of infection to adjoining tissues, although their role in the pathogenesis of disease is not well defined.[16] From 1945 onwards, Penicillin destroying enzymes were encountered, first in strains isolated in hospitals, then those from general population. Four types

of Staphylococcal penicillinase have been described.All types are inducible and extra cellular.

Genome

The Staphylococcal genome consists of a circular chromosome (of approximately 2800 bp), with prophages, plasmids, and transposons. Genes governing virulence and resistance to antibiotics are found on the chromosome, as well as the extra chromosomal elements. These genes are transferred between staphylococcal strains, species, or other gram-positive bacterial species through the extra chromosomal elements.[16] Methicillin-resistant *Staphylococcus aureus* (MRSA) strains possess the mecA gene, which is carried by a unique mobile genetic element, Staphylococcal cassette chromosome (SCCmec), integrated into the *Staphylococcus aureus* chromosome, SCC, whose integration into and excision from the *Staphylococcus aureus* chromosome are mediated by a unique set of recombinase genes, *ccrA* and *ccrB*. A full characterization of MRSA strains requires structure determination of the complex SCCmec element. In addition to the SCCmec types I–V, several subtypes have been isolated. [108,110]

Genetic Regulation of Virulence-Determinant Expression

Global regulatory genes that coordinate the expression of various groups of staphylococcal genes have been identified. The most extensively studied gene, agr, induces the expression of exoprotein (extra cellular protein) while suppressing the expression of surface protein through a bacterial-density–sensing octapeptide. Surface proteins are predominantly synthesized during the exponential growth phase, and the secreted proteins are synthesized during the stationary phase. This sequential expression of genes may have clinical importance. Different stages of staphylococcal infection appear to require different panels of virulence determinants. During the initial stages of infection, the expression of surface proteins that bind extra cellular-matrix molecules favors successful colonization of host tissues, whereas the synthesis of exoproteins favors the spread to adjacent tissues. This hypothesis is supported by studies in animals showing that the inactivation of regulatory genes reduces bacterial virulence[16]

Epidemiology of Staphylococcal Disease

Colonization and Infection

Human beings are a natural reservoir of *Staphylococcus aureus*. Thirty to fifty percent of healthy adults are colonized, with 10 to 20 percent persistently colonized. [17,18] 60% intermittent carriers and 20% non carriers. Both methicillin-sensitive and methicillin-resistant isolates are persistent colonizers and most often involves anterior nares and is frequently asymptomatic. Persons colonized with *Staphylococcus aureus* are at increased risk for subsequent infections. Rates of such colonization is high among patients with type 1 diabetes, intravenous drug users, [19] patients undergoing hemodialysis,[20] surgical patients and patients with the acquired immunodeficiency syndrome. Patients with qualitative or quantitative defects in leukocyte function are also at increased risk for staphylococcal disease. MRSA colonization is particularly important in hospital environment.[22]

Pathogenesis of staphylococcal disease

Transmission

Persons colonized with *Staphylococcus aureus* strains are at increased risk of becoming infected with these strains. Most cases of nosocomial infection are acquired through exposure to the hands of health care workers after they have been transiently colonized with staphylococci from their own reservoir or from contact with an infected patient. Outbreaks may also result from exposure to a single long-term carrier or environmental sources, but these modes of transmission are less common. Air borne transmission may rarely occur in MRSA positive cases with upper respiratory tract infections.[21] One in five chance that a patient harbouring MRSA at admission can go on to develop an infection if not identified and treated.[45]

Temporal Trends in Staphylococcus aureus Disease

The numbers of both community-acquired and hospital-acquired staphylococcal infections have increased in the past 20 years. This trend parallels the increased use of intravascular devices. During the period from 1990 through 1992, *Staphylococcus aureus* was the most common cause of nosocomial cases of

pneumonia and surgical-wound infections and the second most common cause (after coagulase-negative staphylococci) of nosocomial bloodstream infections, according to data from the National Nosocomial Infections Surveillance system of the Centers for Disease Control and Prevention (CDC) [16].

A second trend, resulting in part from selective antibiotic pressure, has been the dramatic worldwide increase in the proportion of infections caused by methicillin-resistant *Staphylococcus aureus*. Initially noted in tertiary care hospitals, methicillin-resistant strains are increasingly found in the community. Data from the National Nosocomial Infections Surveillance system for the period from 1987 to 1997 show that the number of methicillin-resistant *Staphylococcus aureus* infections in intensive care units has continued to increase. Methicillin-resistant strains have also become resistant to other antimicrobial agents.

Staphylococcus aureus has a diverse arsenal of components and products that contribute to the pathogenesis of infection. These components and products have overlapping roles and can act either in concert or alone. A great deal is known about the contribution of these bacterial factors to the development of infection. Considerably less is known about their interaction with each other and with host factors and their relative importance in infection.

The virulence of *Staphylococcus aureus* infection is remarkable, given that the organism is a commensal that colonizes the nares, axillae, vagina, pharynx, or damaged skin surfaces. Infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. Whether an infection is contained or spreads depends on a complex interplay between *Staphylococcus aureus* virulence determinants and host defense mechanisms.

The biology of colonization of the nares, the primary reservoir for staphylococci, is incompletely understood. Mucous membrane appears to be the critical host surface that is colonized in a process involving interactions between staphylococcal protein and mucin carbohydrate. The role of other commensals, secretory IgA, or specific staphylococcal adhesins is unknown.

The risk of infection is increased by the presence of foreign material. Elek and Conen, first demonstrated the ability of sutures to reduce the threshold for infection. Several factors contribute to the increased susceptibility to infection. Phagocytic function in the presence of foreign material in seriously impaired. Devices such as intravenous catheters are rapidly coated with serum constituents, such as fibrinogen or fibronectin, which enable staphylococci to adhere through MSCRAMM-mediated mechanisms and to elaborate glycocalices that further facilitate colonization. Intravenous catheters are frequently implicated in the pathogenesis of nosocomial endocarditis. The introduction of long-term indwelling catheters has led to cases of nosocomial endocarditis that resemble the animal model of endocarditis. The catheter traumatizes the valvular surface, creating a nonbacterial thrombus on the cardiac valve that facilitates subsequent bacterial adherence.[16]

Invasive Infections

Endocarditis, metastatic infection, or the sepsis syndrome may complicate Staphylococcal bacteremia. The endothelial cell is central to these pathogenic processes. Not only is it a potential target for injury, but also its activation contributes to the progression of endovascular disease. Staphylococci avidly adhere to endothelial cells and bind through adhesion–receptor interactions. In vitro studies demonstrate that after adherence, endothelial cells phagocytose staphylococci.

The intracellular environment protects staphylococci from host defense mechanisms as well as the bactericidal effects of antibiotics. Vesga et al demonstrated that the intraendothelial-cell milieu fosters the formation of small-colony variants. These factors may enhance bacterial survival and contribute to the development of persistent or recurrent infections. Staphylococcal strains that cause endocarditis adhere to both damaged and undamaged native valvular surfaces, are resistant to platelet microbicidal proteins, [23]and elaborate proteolytic enzymes that facilitate spread to adjacent tissues. The adherence of staphylococci to the platelet–fibrin thrombus that forms on damaged valvular surfaces may involve the adherence of MSCRAMM proteins to exposed matrix molecules. Staphylococcal endocarditis also occurs on undamaged valves. The invasion of endothelial cells by

Staphylococcus aureus may initiate the cellular alterations, including the expression of tissue factor, that promote the formation of vegetations

Toxin-Mediated Disease

Pyrogenic-toxin superantigens cause life-threatening disease that is characterized by the rapid onset of high fever, shock, capillary leak, and multiorgan dysfunction. Super antigens are T-cell mitogens that bind directly to invariant regions of MHC class II molecules, bypassing intracellular protein ingestion and digestion and subsequent peptide presentation by antigen-presenting cells. The MHC-bound super antigens then attach to T cells according to the composition of the variable region of the T-cell–receptor chain. Toxic shock syndrome toxin 1 binds all variable-region 2–positive T cells, causing an expansion of Clonal T cells (5 to 20 percent of resting T cells as compared with 0.01 percent of T cells for processed antigens), resulting in the massive release of cytokines by both macrophages and T cells. These cytokines mediate the toxic shock syndrome, whose pathophysiology mimics that of Endotoxic shock. In both syndromes, bacterial products induce the release of excessive quantities of cytokines, which then cause tissue damage.[16]

Host Response to Infection

The typical pathological finding of staphylococcal disease is abscess formation. Leukocytes are the primary host defense against *Staphylococcus aureus* infection. The migration of leukocytes to the site of infection results from the orchestrated expression of adhesion molecules on endothelial cells. This cytokine-mediated process is triggered by bacteria and tissue-based macrophages. After infection, cytokines are first demonstrable within vessels, extending into the tissues, as inflammatory cells migrate to the sites of infection. *Staphylococcus aureus* infected endothelial cells also express intercellular adhesion molecule 1 (CD54), vascular-cell adhesion molecule 1 (CD106), and MHC class I molecules and probably contribute to this process. [24]Genetically manipulated mice lacking intercellular adhesion molecule1 have a defect in leukocyte migration that results in increased mortality, but they also have less severe staphylococcal infections than normal mice, perhaps as a result of decreased leukocyte-mediated damage.[25]

The presence of opsonizing antibody directed against capsule, peptidoglycan, or complement facilitates Phagocytosis in vitro. The role of antibody in vivo is less certain, since the titer of antistaphylococcal antibodies is not correlated with protection from infection, except in the case of toxic shock syndrome, in which the presence of anti–toxic shock syndrome toxin 1 is protective. At present, it is not known which staphylococcal components are capable of inducing protection from subsequent infection.

Diseases Caused by *Staphylococcus aureus*

Staphylococcus aureus infection is a major cause of skin, soft-tissue, respiratory, bone, joint, and endovascular disorders. The majority of these infections occur in persons with multiple risk factors for infection. *Staphylococcus aureus* causes a variety of skin infections ranging from superficial skin infections to severe, toxin mediated systemic infections. *Staphylococcus aureus* produces many extracellular products, including toxins that affect host cell function or morphology[42]

Folliculitis

Follicultis is a benign infection restricted to the ostia of the hair follicles and is characterized by the presence of small reddish painful lesions [3, 5]

Furuncles &Carbuncle

Furuncles are deep-seated pyodermas that are present as painful firm, raised lesions with necrotic centers containing purulent material. Carbuncles refer to even more deep-seated lesions that involve the subcutaneous tissues. Several lesions may be present and may coalescence via formation of subcutaneous tracts. Carbuncles are often associated with systemic signs of chills and fever.[3, 5]

Impetigo

It is a superficial staphylococcus infection that is seen mostly in children and usually present on exposed areas especially on the face. Impetigo begins as red macules that evolve into vesicles containing serosanguinous fluid. The lesions

eventually rupture becomes dry and crusted with honey coloured scab on an erythematous margin. *Staphylococcus aureus* accounts for 80-90% of cases and rest contributed by *Group A Streptococcus* [3, 5]

Cellulites and Erysipelas

Cellulites and erysipelas are terms for diffuse, spreading skin infections without underlying purulence or necrosis (tissue death). The terms are often used interchangeably but, classically, erysipelas is defined by the fact that the lesion is raised above the level of the surrounding skin and that there is a clear line of demarcation between involved and uninvolved tissue. Cellulites, on the other hand, are not so clearly demarcated and tend to involve the deeper skin tissues and subcutaneous fat. Both may also be associated with lymphangitis (inflammation of the lymph vessels manifest by "streaking" erythema, or redness, extending from the lesion) and swelling of regional lymph nodes. Often, the skin can take on a brawny, wooden texture. Superficial blisters may develop, as well. Systemic symptoms, such as fever, rapid heart rate, and low blood pressure, can often accompany erysipelas or cellulites and are signs of serious illness that requires prompt evaluation and treatment with systemic antibiotics. In less than 5% of all cases, the infecting organism can be isolated from the blood stream.[3, 5, 16]

Staphylococcus scalded skin syndrome

Staphylococcal toxic shock syndrome (STSS) and staphylococcal scalded skin syndrome (SSSS) are 2 distinct toxin mediated diseases with very distinct cutaneous features. STSS was first described in 1978 and TSST-1 and enterotoxins are associated with it .[88] Staphylococcal Scalded Skin Syndrome was produced by exfoliative toxins A and B which were first described in 1975 .In this, bullous formations occur over large areas of the body with subsequent sloughing of the superficial skin layers. This result in the exposure of large areas of denuded and raw skin .The disease is usually seen in neonates and infants. *Staphylococcus aureus* is not present in the skn lesions but is confined to the primary site of infection, usually the nasopharynx.[63]

Staphylococcal toxic shock syndrome came to prominence in 1980–1981, when numerous cases were associated with the introduction of super absorbent tampons for use during menstruation. The disease is characterized by a fulminating onset, often in previously healthy persons. The diagnosis is based on clinical findings that include high fever, erythematous rash with subsequent desquamation, hypotension, and multiorgan damage. Alternative diagnoses, including Rocky Mountain spotted fever, streptococcal scarlet fever, and Leptospirosis, must be ruled out. The toxic shock syndrome often develops from a site of colonization rather than infection. Although toxic shock syndrome toxin 1 accounts for more than 90 percent of cases of the syndrome that are associated with tampons, other enterotoxin account for 50 percent of cases unrelated to menstruation. Such cases have increased in the recent past, accounting for approximately one third of all cases and are associated with localized infections, surgery, or insect bites.[16]

Surgical Site Infections (SSI)

Surgical site infections (SSI) account for 12.3%-24% of hospital acquired infections. Overall *Staphylococcus aureus* is the dominant species in SSI followed by enterobacteria. [29] UK surveillance data shows that approximately two thirds of staphylococcus SSI is caused by MRSA. Even higher MRSA prevalence is seen in subspecialties such as vascular surgeries. In great majority of cases, the origin of bacteria appears to be patients own body flora. Few more studies also reported that maximum number of MRSA were recovered from post operative surgical site infections.[28, 29,61] Infections of surgical wounds are the most common adverse events in patients who are hospitalized and have undergone surgery, accounting for 38% of all hospital-acquired infections in surgical patients Superficial incisional infections do not extend below the subcutaneous space and are notable for purulent drainage from the incision, pain, tenderness, swelling, and redness. They typically occur within 30 days of the initial surgery. A deep incisional infection extends into the underlying fascia and muscle. It presents in a similar fashion as the superficial incisional infection and also typically arises within 30 days of initial surgery. However, if prosthesis was inserted, deep incisional infections can occur up to a year after initial surgery. Any deep incisional infection that does not resolve as expected raises the possibility that, in fact, the incisional infection may just be a

superficial manifestation of a deeper organ/space infection. The most common organism is staphylococcus (20%) and incidence of MRSA among surgical site infections (SSI) is increasing.[29]

Factors most consistently associated with increased incidence of post operative infections are over 60 years of age, pre- operative stay in hospital, long duration of surgical procedure, pre-existing infection at site of the wound. Other equally important condition that plays an important role are diabetics, immune suppression, irradiation, mal-nutrition. More recently the extensive use of indwelling medical devices and possibly as a result of introduction of new antibiotic, coupled with their indiscriminate use ,these staphylococci has now emerged as the most predominant pathogen.

Diabetic wound infections

In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) pathogens have been isolated with increasing frequency (30-50%) from wound and skin infections that commonly affect the lower extremities of patients with diabetes. [25,105]For these individuals, foot infections caused by MRSA organisms have been associated with poorer outcomes related to an increased risk of amputations and infection-related mortality.

Burns

Burns provide a suitable site for bacterial multiplication. The clinical consequence of infection in burns may be serious, a large proportion of mortality in burned patients who survived the initial trauma and shock has been due to infection. *Staphylococcus aureus* and *Pseudomonas aeroginosa* are the most common isolates in most burn units. In a study, the incidence of *Staphylococcus aureus* was 41.8%, out of it, 51.6% were MRSA.[1] As in other hospital areas, the emergence of resistant gram positive bacteria including MRSA has been seen in burn units.[30, 31, 33, 34]

Osteomyelitis/Septic arthritis

Staphylococcus aureus osteomyelitis most often occurs as a complication of local infection via direct extension although haematogenous osteomyelitis may

also occur. *Staphylococcus aureus* constitutes to 50-70% of all Osteomyelitis cases. Haematogenous osteomyelitis usually involves long bones in children, but osteomyelitis of vertebral column accounts for 60% of such infection in adults. Abscess and Lytic bone lesion must be aspirated and cultured to provide definitive diagnosis.[3, 5]

Sepsis

A minority of bacteremic or local infections progress to sepsis. Risk factors for sepsis include advanced age, immunosuppression, chemotherapy, and invasive procedures. The presentation of staphylococcal sepsis is similar to that of gram-negative sepsis, with fever, hypotension, tachycardia, and tachypnea. *Staphylococcus aureus* is one of the most common gram-positive pathogens in cases of sepsis. Severe cases progress to multi-organ dysfunction disseminated intravascular coagulation, lactic acidosis, and death. In sepsis, the levels of circulating tumor necrosis factor, interleukin-1, and interleukin-6 are predictive of the outcome. [16] *Staphylococcus aureus* is a much less common cause of neonatal sepsis in recent decades than its peak incidence in 1950s through 1970s.[103] An overall predominance of Gram negative bacteria is seen in these cases nowadays.[104] Localized infections have a tendency to spread to surrounding tissues or frequently via blood stream to distant sites. *Staphylococcus aureus* is commonly isolated from patients with both community acquired and nosocomial infections. They are the second most common cause of nosocomial blood stream infection accounting for 20% of the infections. Incidence varies from 10% in patients under age of one year to 24% in patients over age of 65 years. *Staphylococcus Aureus* infection originates from localized infection, an intravascular catheter or contaminated syringe. The overall rate of mortality from Staphylococcal bacteremia, which has not changed in the past 15 years, ranges from 11 to 43 percent. Factors associated with increased mortality include an age of more than 50 years, irremovable foci of infection, and serious underlying cardiac, neurological, or respiratory disease. Bacteremia caused by methicillin-resistant strains is associated with increased mortality. The change in the Acute Physiology and Chronic Health Evaluation (APACHE II) score from the day before to the day of *Staphylococcus aureus* bacteremia was recently found to predict the clinical course and outcome. The

frequency of complications due to Staphylococcal bacteremia is high, ranging from 11 to 53 percent. As many as 31 percent of patients with bacteremia who do not have evidence of endocarditis, presented with metastatic infection. An increasing percentage of bacteremia infections are related to catheterization. The rate of complications is lower for catheter-related infections than for all cases of bacteremia (24 percent), as is the overall mortality rate (15 percent). Patients with bacteremia or fever that persists for more than 72 hours after the catheter has been removed may have an increased risk of complications.[46] The incidence of endocarditis in patients with catheters, estimated on the basis of clinical indicators, is also low, ranging from 0 to 18 percent. Some studies, however, suggest that the incidence of endocarditis may be higher. Espersen and Frimodt-Møller reported that the diagnosis of *Staphylococcus aureus* endocarditis was made at autopsy and not suspected clinically in 55 percent of the patients in their series (65 of 119). [26] Using transesophageal echocardiography, Fowler et al. recently found that 25 percent of selected patients with staphylococcal bacteremia (26 of 103) and 23 percent of those with catheters as the primary focus (16 of 69) had transesophageal echocardiography evidence of endocarditis in the absence of clinical or transthoracic echocardiography findings.[27]

Endocarditis

The incidence of *Staphylococcus aureus* endocarditis has increased and now accounts for 25 to 35 percent of cases.[16] It occurs in intravenous drug users, elderly patients, patients with prosthetic valves, and hospitalized patients. In all four groups, the initial presentation may be limited to fever and malaise, making diagnosis difficult. Unlike endocarditis caused by less virulent pathogens, *Staphylococcus aureus* endocarditis is characterized by a rapid onset, high fever, frequent involvement of normal cardiac valves, and the absence of physical stigmata of the disease on initial presentation. In one study, 13 percent of febrile intravenous drug users evaluated in an emergency room had endocarditis, and the diagnosis could not have been predicted on the basis of available clinical or laboratory data. In cases of endocarditis related to intravenous drug use, the disease is frequently right-sided, the patients are young, the mortality rate is low, and the majority of patients do not have antecedent valvular disease. The prognosis

is worse for intravenous drug users who have advanced disease associated with human immunodeficiency virus (HIV) infection than it is for those without HIV infection. *Staphylococcus aureus* is one of the most common pathogens in nosocomial and prosthetic-valve endocarditis, and intravascular catheters are the most frequent source of bacterial inoculation. The mortality rate for nosocomial endocarditis, regardless of the pathogen, is 40 to 56 percent, and the rate is even higher when the pathogen is *Stapylococcus aureus.* In many of these cases, the diagnosis is obscured by other conditions or the administration of antibiotics. Prosthetic-valve endocarditis, especially in the early postoperative period, is often Fulminant and is characterized by the formation of myocardial abscesses and the development of valvular insufficiency. Fang et al noted a 43 percent incidence of endocarditis in patients with prosthetic valves who had nosocomial bacteremia. The most common pathogen was *Staphylococcus aureus*. [32]

Pneumonia

Although *Staphylococcus aureus* pneumonia is nosocomial, it accounts for a small proportion of community-acquired pneumonia especially in certain groups like diabetic patients. Although 15-30% of adults are nasal carriers of *Staphylococcus aureus*, resulting pneumonia is rare. Ventilator associated pneumonia especially due to MRSA are rising in incidence (17%) and pose unique challenge in their prevention and treatment. Risk factors for development of MRSA include nasal carriage, poor antibiotic therapy, prolonged mechanical ventilation, poor infection control measures, head injury /coma and viral infections.[35]

Urinary tract infection

Staphylococcus aureus is a relatively infrequent urinary tract isolate in the general population. In a multicenter, community-based study conducted in Great Britain, *Staphylococcus aureus* accounted for only 0.5% of isolates. A similar laboratory-based study conducted in France found that *Staphylococcus aureus* accounted for only 1.3% of isolates from urine specimens submitted from the community. Prior studies suggest that isolation of *Staphylococcus aureus* from the urine is often secondary to staphylococcal bacteremia originating at another site

(e.g., in cases of endocarditis) [36,71] Isolation of *Staphylococcus aureus* from urine samples in the absence of bacteremia is therefore often considered to represent colonization. [37]

In specific patient populations, however, *Staphylococcus aureus* can be an important primary urinary pathogen. For example, MRSA urinary tract infection occurs in both endemic and epidemic fashion among patients undergoing urologic surgical procedures. MRSA bacteriuria occurs among long-term care patients as well, and it is significantly associated with urinary catheterization and antibiotic use.[38] It is problematic to define the exact role of *Staphylococcus aureus* as a cause of symptomatic urinary tract infection, as opposed to colonization, in this population. Long-term care patients have a high frequency of asymptomatic bacteriuria. There is evidence to suggest that the majority of febrile episodes in long-term care patients with bacteriuria are not, in fact, due to urinary tract infection.

Traditionally MRSA has been considered a major nosocomial pathogen. In this decade it has been observed to be emerging in the community as well. The first case of community acquired MRSA infection in the United States was reported in 1980.[43] More widespread identification of community acquired methicillin resistant *Staphylococcus aureus* in the United States began in the year1990, following the report of community acquired methicillin resistant *Staphylococcus aureus* infections among four children. Patients with community acquired methicillin resistant *Staphylococcus aureus* have often lacked the risked factors known for patients with hospital associated MRSA, which include recent hospitalization, dialysis, nursing home residence and other co-morbid conditions such as diabetes, chronic renal failure, chronic pulmonary disease which bring them into contact with health care setting. Community acquired methicillin resistant *Staphylococcus aureus* has also been found to be composed of more diverse clonal groups than hospital acquired methicillin resistant *Staphylococcus aureus*. Earlier only 2% of *Staphylococcus aureus* clinical isolates had panton valentine leukocidin (PVL) gene. The PVL gene has been virtually found in all isolates of community acquired methicillin resistant *Staphylococcus aureus* causing epidemics worldwide.[44] New strains of *Staphylococcus aureus*

displaying unique combinations of virulence factors and resistant traits have been associated with high morbidity and mortality in the community.

The substantial increase in community acquired methicillin resistant *Staphylococcus aureus* infection has increased the challenge of selecting empirical antimicrobial treatment in outpatient settings. Previous studies have reported that in the United States the prevalence of community acquired methicillin resistant *Staphylococcus aureus* infection varies from 76 % among MRSA skin and soft tissue infection isolates in Alaska[65] to 12 % of all MRSA infection in Minnesota.[66] In other studies community acquired methicillin resistant *Staphylococcus aureus* infection rate ranges from 30.2 to 45%. [43] In a study done in India the total Incidence of MRSA was 51.6 % in burns and ortho wards, out of which hospital acquired methicillin resistant *Staphylococcus aureus* was contributed by 87.3 % and community acquired methicillin resistant *Staphylococcus aureus* infection was 12.7 %. [1]

Overall, community acquired methicillin resistant *Staphylococcus aureus* infection was unlikely to result in prolonged hospitalization. Most of these infections responded to wound care and out patient oral antimicrobial therapy. Both hospital acquired *Staphylococcus aureus* and community acquired methicillin resistant *Staphylococcus aureus* infection are highly virulent strains capable of Clonal dissemination and have the ability to cause epidemics of furunculosis and other skin and soft tissue infections. Nevertheless non- health care MRSA are now responsible for majority of community onset MRSA in USA. Furthermore despite their designation as community MRSA strains, these strains are no longer restricted to that setting since community onset MRSA strains have now been found in association with nosocomial infection. Irrespective of the characteristics of population or the setting, the community onset MRSA carrying the SCC mec type IV and V element poses a serious threat and in all likelihood will continue to emerge as a major health concern.[55,56]

Infections Common In Coagualse Negative Staphylococcus

Bacteremia:

The blood stream infection (BSI) caused by CONS is mostly nosocomial but CONS have also been isolated from about 8% of patients with community acquired blood stream infection.[45] The number of cases of nosocomial bacteremia due to coagulase negative staphylococci has been estimated at 50,000-120,000 per year in the United States. In the NNIS data sheet, these bacteremia constitute 8% of all nosocomial infections, but it is very important to differentiate contaminants from infection especially blood samples. Bacteremia due to CONS is nosocomial in origin and is related to presence of an indwelling foreign body. According to several surveys CONS is the most prevalent pathogen isolated from blood accounting for 15-30% of blood cultures isolates.[40,45,59,60] *Staphylococcus epidermidis* is the most frequently isolated species among CONS has been implicated in a variety of infections including blood stream infection [39]mostly associated with intravascular catheters and other prosthetic devices. *Staphylococcus haemolyticus*, the second most commonly isolated species.[9]A variety of other CONS have also been implicated in BSI including *Staphylococcus capitis, Staphylococcus homins, Staphylococcus lugdenesis, Staphylococcus saprophyticus, Staphylococcus schleiferi, Staphylococcus simulans* and *Staphylococcus warneri*. The proportion of cons among blood culture isolates seem to decrease with increasing age.[40] Crude mortality in patients with CONS blood stream infection ranges from 14 to 31%[40]

Catheter related infections

Staphylococcus epidermidis is responsible for 50-70% of these infections. Coagulase negative Staphylococcal bacteremia is seldom life threatening especially if treated promptly, although frank sepsis syndrome may occur especially in immunocompromised patients. Coagulase Negative Staphylococci may produce exit -site infections, tunnel infections, infected thromobophlebitis and other complications such as infective endocarditis and abscesses. *Staphylococcus lugdenensis* has also been implicated in catheter related infections, arthritis and prosthetic joint infections.[41]

Central nervous system shunt infections

Coagulase Negative Staphylococci are the most common bacteria isolated from cerebrospinal fluid shunt infections {48% to 67% of isolates) and *Staphylococcus epidermidis* is the predominant species.[3, 5]

Endocarditis

Native valve endocardits due to Coagulase Negative staphylococci is relatively rare and is characterized by a relatively indolent clinical course. *Staphylococcus epidermidis* is the most common species. Other species such as *Staphylococcus warneri* and *Staphylococcus lugdunensis* are also implicated in infections of prosthetic valves in 40-50% of cases. [3, 5]

Urinary tract infections

Urinary tract infections due to *Staphylococcus saprophyticus* mainly affect young female outpatients[47]. It is implicated in 10-20% of UTI in females. It is 2nd most common cause of UTI in women between ages 17-27 years. Nosocomial urinary tract infections due to other staphylococci mainly *Staphylococcus epidermidis* occurs equally in men and women, especially in those with urinary catheters.[3, 5]

Surgical site infection (SSI)

The most common organism causing SSI is *Staphylococcus aureus* (20%) and SSI caused by CONS is 14% and *Enterococci* contribute to 12%[29]. CONS are ranked second according to NNIS data. Most infections are mainly caused by patients own endogenous skin flora

Other common infections that can occur by CONS are bone and joint infections, opportunistic infections and wound infections, mainly by *Staphylococcus haemolyticus*.

Identification of staphylococcus aureus and other coagulase negative staphylococci

Collection and handling of specimens

Proper collection, transport and processing are essential in the correct diagnosis and interpretation of any bacterial culture result. Clinical materials collected from infected sites should be transported to the laboratory without delay to prevent drying, maintain the proper environment and minimize growth of contaminating organisms [4, 5]

Stuart's and Amie's gel transport media can be used for transport of samples. There are studies which say that Stuart's media is better than Amie's gel media[106]

Microscopic examination and culture:

Cultures of clinical specimens should be performed as soon as possible to minimize changes in the microbial composition from what was originally present. Direct microscopic examination of normally sterile fluids may provide a rapid presumptive report of gram-positive cocci resembling staphylococci. Isolation of staphylococci from primary clinical specimens like pus, wound swabs, CSF, joint aspirates, urine samples, blood cultures etc is usually performed using blood agar and Maconkey agar, following an incubation period of 18-24 hour in air at 35-37◦C. Colonies are usually 1-3 mm in diameter, circular, smooth and raised with butyrous consistency. Colonies of most species and sub species cannot be differentiated from one another on this medium. Pigmentation is best seen on milk agar. Screening for the presence of *Staphylococcus aureus* in mixed cultures such as nasal swabs is often performed using mannitol salt agar. *Staphylococcus aureus* ferments mannitol resulting in a change in the colour of the medium from pink to yellow.[4, 5, 11]

Identification of *Staphylococcus aureus*

Staphylococcus aureus is distinguished from other Staphylococcal species on the basis of their yellow or cream pigmentation of colonies and positive results of Coagulase, Mannitol-fermentation, and deoxyribonuclease tests.[13] *Staphylococcus aureus* is often beta hemolytic on blood agar, Staphylococcus *epidermidis* is non hemolytic. Staphylococcus *aureus* are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid.

They are separated from streptococci by the catalase test. Streptococci are catalase negative. This test is performed by adding 3% hydrogen peroxide to a colony on slide or a tube. Catalase positive cultures produce oxygen and bubble at once. Staphylococcus *aureus* is differentiated from CONS by the coagulase test. Free Coagulase clots plasma (preferably rabbit's) in the absence of calcium.

Coagulase test:

Staphylococcus aureus produce a coagulase ,a prothrombin activator and convert fibrinogen to fibrin. Most strains produce both free coagulase which react in the tube coagulase test and bound Coagulase (clumping factor) which react in the slide coagulase test.Asmall minority of strains form only the one or the other type of coagulase.Lesser common species namely *Staphylococcus schleiferi and Staphylococcus* lugdunensis may also give slide coagulase positive[4,5].

Fermentation of mannitol is another important feature of *Staphylococcus aureus*. Mannitol salt agar (MSA) or variations of this medium have been extensively used as a screening medium for *Staphylococcus aureus* especially MRSA .The reported sensitivity of this media has varied widely [51].The addition of lipovitellin to detect lipase production may markedly increase the sensitivity of MSA

The most prevalent staphylococcal species and subspecies in human infections are Staphylococcus aureus, *Staphylococcus epidermidis Staphylococcus haemolyticus* and *Staphylococcus saprophyticus* followed by *Staphylococcus*

hominis Staphylococcus warneri, Staphylococcus lugdunensis, Staphylococcus schleiferi Staphylococcus capitis and *Staphylococcus simulans.* [9] *Staphylococcus haemoltyicus* has been documented as a cause of primary and nosocomial bacteremia, wound and soft tissues infections, urinary tract infection and nosocomial pediatric and neonatal infections. *Staphylococcus hominis* found on skin and occasionally isolated from infections as a low-grade pathogen-causing catheter related sepsis in immunocompromised host. *Staphylococcus simulans* found on skin and in urethra of healthy women. This organism has been isolated as a cause of septicemia, osteomyelitis, native valve endocarditis, and septic arthritis following open reduction of fractured fibula, vertebral osteomyelitis and prosthetic joint infections. *Staphylococcus cohini* was first described in 1975 and 1991.*Staphylococcus cohini subspecies cohini* has been isolated in humans. Though normal flora of the skin it is an emergent opportunistic agent having been reported as a cause of community acquired pneumonia, septic arthritis, catheter related sepsis, chorioaminontis and neonatal sepsis with meningitis. The above staphylococcal species and sub species can be distinguished on the basis of minimal key characters. Speciation of CONS is done according to method followed by Kloos and S chleifer.[77] The test includes fermentation of sucrose, trehalose, maltose, lactose, xylose, mannose, fructose and mannitol. Other test like VP test for looking for production of acetoin, urease test, polymyxin B sensitivity, ornithine decarboxylase test, nitrate reduction, phosphatase test on phenophathalein diphosphate agar and susceptibility to novobiocin.

Mechanisms of Resistance to Antimicrobial Agents

Penicillin is inactivated by beta-lactamase, a serine protease that hydrolyzes the beta-lactam ring. Less than 5 percent of isolates remain sensitive to penicillin[16]. In a study, incidence of Methicillin resistant *Staphylococcus aureus* was 31%, and almost all strains (99.6%) were resistant to penicillin, 93.6% were resistant to Ampicillin in addition to 63.2% resistance towards gentamicin, cotrimoxazole, erythromycin and cefotaxime.[59] Some other studies quote 100%resistance to penicillin in addition to 97% resistance to cotrimoxazole and 93.3%resistant to chloramphenicol.[67]

Resistance to methicillin confers resistance to all penicillinase-resistant penicillins and cephalosporins. This high level of resistance requires the presence of the mec gene that encodes penicillin-binding protein 2a. The mec genes probably originated from a different species of staphylococci. Although many methicillin-resistant strains appear to be descendants of a limited number of clones, some appear to be multiclonal in origin, suggesting the horizontal transfer of mec DNA. Other staphylococcal genes, including bla (for beta-lactamase) and fem (for factors essential for methicillin resistance), affect the expression of resistance. The expression of resistance to methicillin is often heterogeneous, and the percentage of a bacterial population that expresses the resistance phenotype varies according to the environmental conditions. The first methicillin resistant staphylococci was first reported in the United Kingdom in 1961,shortly after methicillin was introduced into clinical practice.[6, 7] Seven years later after the resistant strain had become widespread in Japan, Europe, and Australia, the first case of MRSA was described in the United states in1968. Antimicrobial-sensitivity testing has been modified to enhance the detection of the resistance phenotype. . Confirmation of sensitivity by the agar or broth-dilution method is recommended. Other methods for detecting MRSA include, use of oxacillin resistance screen agar, determination of mic to oxacillin, detection of penicillin binding protein(PBP2a) by using latex agglutination, detection of Mec Agene and Fem A/B gene etc by PCR.

Resistance to Vancomycin

Resistance to vancomycin has been reported in clinical isolates of *Staphylococcus haemolyticus,* a coagulase-negative species. The enterococcal plasmid-bearing gene for resistance to vancomycin has been transferred by conjugation to *Staphylococcus aureus* in vitro. Three types of resistance to vancomycin have been described since1997 in Staphylococci.

i. *Staphylococcus aureus* with intermediate level resistance to vancomycin (VISA)
ii. *Staphylococcus aureus* with hetero resistance to vancomycin (hVISA)
iii. Vancomycin resistant *Staphylococcus aureus* (VRSA)

The main mechanism of vancomycin resistance for VISA and VRSA are currently under investigation.

Four recent case reports (one from Japan and three from the United States) have documented the isolation of clinical strains with intermediate sensitivity to vancomycin (minimal inhibitory concentration, 8-16 µg per milliliter). [3]The mechanism of resistance in these isolates is not known but is not due to the van genes present in enterococci. Both increased cell-wall synthesis and alterations in the cell wall that prevent vancomycin from reaching sites of cell-wall synthesis have been suggested as mechanisms. Screening for strains of *Staphylococcus aureus* with intermediate sensitivities to glycopeptides including vancomycin (glycopeptide-intermediate strains) can be performed with the use of brain–heart infusion agar plates supplemented with 6 µg of vancomycin per milliliter. The incidence of vancomycin intermediate staphylococcus aureus and vancomycin resistant *Staphylococcus aureus* has been increasing in various parts of the world.[101,102] Vancomycin-resistant *Staphylococcus aureus* strains are likely to pose a major therapeutic challenge in the future.

Resistance to macrolides, lincosamide and streptogramin (MLS) [3]

Erythromycin resistant staphylococci often have cross resistance to macrolides, lincosamides and streptogramin type B antibiotics (designated MLS resistant) The different types of MLS antibiotics bind to 50 s ribosomal subunit at overlapping binding sites and binding interferes with transpeptidation and translocation needed for peptide chain elongation. Clinical isolates of constitutive MLS resistant staphylococci are continuing to increase in frequency and this trend may be a reflection of increased clinical use of clindamycin. The incidence of erythromycin resistance is about 63% in MRSA and 34.4% in MSSA.[57]In another study incidence was about 25.3%[58]. Erythromycin resistance through the erythromycin ribose methylase gene (erm gene) and initially susceptible to clindamycin can potentially develop resistance to clindamycin during therapy. To detect inducible clindamycin resistance 15µg erythromycin and 2µg clindamycin disk were placed 15 -20 mm apart for *Staphylococcus aureus* and 20-26mm for coagulase negative staphylococcus strains. *Staphylococcus aureus* 25923 was used as control for these tests. In the double disk diffusion test, if there is

inducible clindamycin resistance, the erythromycin will diffuse through out the agar, and resistance to lincosamide will be induced resulting in flattening or blunting of the lincosamide zone of inhibition adjacent to the erythromycin disc giving the shape of D to the zone. The isolates resistant to both erythromycin and clindamycin were defined as showing constitutive MLSb resistance and those that showed the flattening between the two disks was defined as having inducible MLSb resistance. The rate of induced clindamycin resistance in MRSA isolates vary widely from 2- 8% in Houston TX to 94% in Chicago. There are studies quoting 30% induced clindamycin resistance.[94] So nowadays many laboratories perform double disk diffusion test (Dtest) to determine whether Clindamycin susceptible MRSA habours inducible resistance.

The inducible macrolide and streptogramin resistance phenotype involves erythromycin cross-resistance to other 14-16 membered ring macrolides and streptogramin type B, but not the lincosamides.[3]

Resistance to tetracycline

This is wide spread among staphylococcal species and ranks, and along with Beta lactam and MLS resistance is one of the most frequent antibiotic resistance found in natural populations of staphylococci. The incidence of Tetracycline resistance in MRSA ranges from 33.5 to 63.1% and in MSSA it ranges from 22.4 to 27.5%.[3,4,107] There are two mechanisms of tetracycline resistance; the most common one is the energy dependent pumping (efflux) of tetracycline and doxycycline from the cells, so that levels of these antibiotics are reduced below the required, to inhibit the ribosome. The efflux protein is most often coded by the inducible gene tetK which is located on class I plasmids of the pT181 family. The second mechanism, one that is controlled by gene Tet M involves ribosome protection such that protein synthesis is unaffected by the presence of tetracycline, doxycycline or minocycline.[3]

Resistance to aminoglycosides

In studies done on antibiotic susceptibility of MRSA, the incidence of resistance to amikacin is about 52.6%[59] and resistance to gentamicin ranged between 96.7% to 100% [60,61,62,63]

There are three major mechanisms for aminoglycoside resistance[3]

i. The Change in ribosomal proteins as a consequence of certain mutations in their structural genes such that ribosomes can no longer bind streptomycin.
ii. Energization and permeability of the cell membrane.
iii. Modification of aminoglycosides by aminoglycoside - modifying enzymes so that antibiotics are no longer capable of binding to ribosome. The genes encoding these enzymes are located on plasmids (eg gentamycin resistance plasmids) or on the chromosomes

Resistance to trimethoprim

It is mediated by alteration in the expression of the intrinsic chromosomal dfr gene possibly resulting in the overproduction of the native dihydrofolate reductase (DHFR) or a reduced affinity of native DHFR for trimethoprim or by acquisition of a second chromosomal or plasmid dfr gene that encodes a trimethoprim resistant DHFR capable of rescuing the reduction step leading to tetrahydrolate in the presence of Trimethoprim.The incidence of resistance to cotrimoxazole ranges between 8.5% and 70%[59,60]

Resistance to fluroquinolones

The incidence of resistance to ciprofloxacin in MRSA ranges from 86.1% to 92.8%[59]

There are three mechanisms by which resistance is developed[3]

i. Mutation in the gyrA encoding the DNA gyrase subunit A

ii. A second mechanism involves mutations in the chromosomal gene Nora or its regulatory region that encodes a membrane efflux protein for hydrophilic fluroquinolones and other unrelated antibiotics

Iii A third mechanism involves mutation in chromosomal gene grlA that encodes the A subunit of DNA topoisomerase IV

Detection of Methicillin resistance by different methods

Disc diffusion methods[49, 50,86]

Disc diffusion methods remain the mostly widely used in routine clinical laboratories. Traditionally oxacillin has been tested and results are representative of all beta lactam agents. Cefoxitin has been recently investigated as an alternative agent for detection of resistance by disc diffusion and all studies indicate that the test is more reliable than those with oxacillin disc.

Mannitol salt agar medium (MSA) [49, 50]

MSA was developed in 1945 as a selective medium for isolation of pathogenic staphylococci. It is regarded as a valuable medium for isolation of *Staphylococcus aureus* from water, milk, skin, respiratory tract secretion and nose. Since 1985, MSA has been studied with regards to its suitability as a medium for susceptibility testing. In a particular study, disc diffusion tests using 1µg of oxacillin on MSA was found to be an excellent screening method to detect oxacillin resistance in *Staphylococcus aureus*, giving a sensitivity of 100% and a specificity of 97.6%[51].The agar screen test on MSA was highly sensitive (98.1%) and specificity was 95%. [51]

Lipovitellin salt mannitol agar[50]

This medium has also been evaluated for disc diffusion tests with 1µg of oxacillin. A zone diameter of <13mm was interpreted as evidence for oxacillin resistance. This medium has a sensitivity of 100 % and a specificity of 70%.

Dilution methods

Agar dilution: [50] **–** Test on Muller Hinton Agar with 2% Sodium chloride and an inoculum of 10^4 cfu/ml will distinguish most resistant from susceptible strains. This method requires incubation for 24 hours at 33-35 ºC. These are among the NCCLS recommendations for increasing the chance of detection of resistance by agar and broth dilution.In this method an excelling MIC <2 mg/L indicates that the strain is susceptible and an MIC > 2mg/L indicates that the strain is resistant.

Broth microdilution.[50]

This method recommended by NCCLS requires the use of Mueller Hinton broth with 2% sodium chloride, an inoculum of $5*10^5$ cfu/ml and subjected to incubation at 33-35 C for 24 hours.

E test method [50]

The E test method using Mueller Hinton agar with 2%sodum chloride, an inoculum density equivalent to 0.5- 1.0 McFarland standard, an application of inoculum with a swab and incubation at 35◦ C for 24 hours.

Agar screening method [52]

The method recommended by NCCLS requires suspending the test organisms to a density of 0.5 Macfarland standard and inoculating into Mueller Hinton agar containing 4% sodium chloride and 6mg/l oxacillin with a spot or streak of the organisms. Plates are incubated at 35◦ C or less for 24 hours and any growth other than a single colony indicative of resistance. This method has been recommended for screening colonies isolated on routine media and for confirmation of suspect resistance seen in disc diffusion tests.

Latex agglutination [50, 53]

A rapid slide agglutination based on detection of PBP2a is commercially available. This method involves extraction of PBP2a from a suspension of

colonies and demonstration of agglutination with latex particles coated with monoclonal antibody to PBP2a. There are studies done and test was proved to be highly sensitive and specific.[89, 90]

Molecular methods[50]

Detection of genes specific for resistance, that is, the MecA gene for methicillin resistance of Staphylococcus aureus is done by Polymerase Chain Reaction (PCR). As of now this method is the Gold standard for the detection of MRSA. This has become a gold standard for comparison of sensitivity and specificity of other test. Some investigators include PCR detection of second gene such as fem A, Fem B and a specific marker gene nuc specific for *Staphylococcus aureus*, and multiplex PCR can be performed to detect different genes associated with resistance to different antibiotics groups [54]

Bacteriophage typing[11]

Bacteriophage typing is essential in the detection of sources of nursery epidemics and other outbreaks and for controlling such events.It is based on the susceptibility of *Staphylococcus aureus* to infection by various bacterial viruses (bacteriophages). The set of basic phages used for *Staphylococcus aureus* are

Group 1 - 29, 52, 52A, 79, 80

Group2 – 3A, 3B, 3C, 55, 71

Group 3- 6, 7, 42E, 47, 53,54,75,77, 83A

Group 4- 42D

Miscellaneous -81,187

Phage typing has been used to type isolates of *Staphylococcus aureus* for over 45 years. Various molecular methods have been described for typing isolates of MRSA. They include ribotyping, random amplification of polymorphic DNA by PCR insertion sequence profiling, PCR-restriction fragment length polymorphism (PCR-RFLP) and, notably, pulsed-field gel electrophoresis (PFGE) [109]

Treatment of *Staphylococcus aureus* Infection

Penicillin remains the drug of choice if the isolate is sensitive to it. Semi synthetic penicillin (nafcillin or cloxacillin) is indicated for beta-Lactamases–producing strains. In patients with histories penicillin allergy, a cephalosporin such as cefazolin or cephalothin is an acceptable alternative. Changes in pattern of antimicrobial susceptibility of *Staphylococcus aureus* have been reported world wide especially in developing countries, making antimicrobial agents increasingly, less effective in treating bacterial infections. Over the past twelve years there have been dramatic changes in the susceptibility of *Staphylococcus aureus* in both hospitals acquired and community acquired infections. The hospital acquired MRSA(HAMRSA) is more multi drug resistant compared to community acquired MRSA (CAMRSA). The older β lactams, penicillin and Ampicillin are ineffective against more than 80% of strains and resistance to many of the non β lactam agents such as tetracycline, erythromycin and Clindamycin has gradually reached alarming levels by the year 1990. MRSA and MSSA strains can easily spread from infected patients to medical staff that often becomes transient carriers. An important component of therapy, especially when cutaneous community acquired methicillin resistant *Staphylococcus aureus* infection presents as abscess, is the incision and drainage. In addition to this, systemic antibiotic therapy can be given. Topical applications such as mupirocin can be given for decolonization of intranasal MRSA and other carriers. CAMRSA are more susceptible to other antibiotics, such as ciprofloxacin and Clindamycin. Studies show that there is usually a high prevalence of resistance to erythromycin (61%- 93%). [64, 65]

Trimethoprim- sulphamethoxazole can be given as monotherapy or in combination with rifampicin to treat community acquired methicillin resistant *Staphylococcus aureus* especially those of cutaneous orgin ones. Clindamycin is another drug which can be given in combination with rifampicin is also useful. But disadvantage is that it can cause pseudomembranous colitis.In addition increasing Clindamycin resistance is emerging recently. Community acquired methicillin resistant strains that are Erythromycin resistant through the erythromycin ribose methylase gene (erm gene) and initially susceptible to Clindamycin can potentially develop resistance to Clindamycin during therapy. The rate of induced

Clindamycin resistance in MRSA isolates vary widely from 2- 8% in Houston TX to 94% in Chicago.So nowadays many laboratories perform double disk diffusion test to determine whether Clindamycin susceptible MRSA harbors inducible resistance. MRSAs may be susceptible to tetracycline .The susceptibility to MRSA especially community acquired methicillin resistant to fluroquinolones is variable. Newer quinolones like gatifloxacin and moxifloxacin if used in combination with other antibiotics may not susceptible to development of resistance by community acquired methicillin resistant staphylococcus aureus. . Serious infections, vancomycin I/V is recommended, however subsequent culture confirmation of MRSA is suggested, as β lactams such as cefazolin and semisysthetic penicillanase resistant penicillin such as oxacilin, naficillin are rapidly more bacteriocidal and more efficacious than vancomycin against MSSA. The MRSA are usually also resistant to other β lactams, so infections with MRSA are life threatening especially in immunocompromised often becoming difficult to eradicate. Vancomycin is the drug of choice for methicillin-resistant isolates. Patients unable to tolerate vancomycin have been treated with fluoroquinolones, trimethoprim–sulfamethoxazole, clindamycin, or minocycline. Each of these drugs has been effective in cases that require bactericidal therapy. They are not as effective as vancomycin, however, either because they have less antistaphylococcal activity or because resistance develops during therapy. Quinolones with enhanced antistaphylococcal activity have recently become available, but their use may also be limited by the development of resistance during therapy. A number of potentially active drugs are under investigation, including quinupristin–dalfopristin, a new carbapenem, and a new family of antimicrobial drugs, oxzolidinones. Several new staphylococcal agents have recently been developed that provide additional therapeutic alternatives for patients with MRSA such as linezolid, quinpristin-dalphopristin, teicoplanin and Tigecyline .In addition emerging agents that are currently being evaluated and may be useful for treating MRSA include ceftobiprole, dalbavancin, oritavancin and telavancin. Antimicrobial combinations have been used to increase bactericidal activity or to prevent the development of antimicrobial resistance. The combination of beta -lactams and aminoglycosides increases bacterial killing in vitro and in animal models of endocarditis. In a clinical trial comparing a single

drug with combination therapy for the treatment of endocarditis, combination therapy resulted in more rapid clearance of bacteria from the bloodstream, but the clinical outcome was the same with the two approaches. Many clinicians use an aminoglycoside, when possible, for the first few days of therapy.

Rifampicin is another potent antistaphylococcal drug, but resistance invariably develops if it is used alone. Although the efficacy of rifampin as an adjunctive drug in patients with life-threatening infections remains controversial, it is recommended in combination with gentamicin and vancomycin or nafcillin for the treatment of prosthetic-valve endocarditis. Rifampicin has also been combined with quinolones in an effort to prevent the development of resistance

The duration of therapy for invasive, life-threatening infections, including those that cause endocarditis, osteomyelitis, or arthritis, is four weeks or longer. The appropriate duration of treatment for bacteremia originating from a removable focus of infection, such as an intravascular catheter, is controversial. A two-week period of therapy has been recommended for infections considered to pose a low risk of complications (those caused by catheterization in non-immuno compromised patients without valvular abnormalities, with prompt removal of the catheter, rapid clearance of bacteremia, and no evidence of metastatic infection). However, a meta-analysis concluded that, despite the reportedly low complication rates, the available data do not justify short-course therapy in such patients. There is also concern that endocarditis has been under- diagnosed because of a reliance on clinical criteria. Despite some promising studies, tests for serum anti–teichoic acid antibodies to help identify patients at risk for complications have not proved useful.

Prevention of Staphylococcal Disease

The revised guidelines for control of MRSA include isolation of patient in a separate cubicle, limiting the movement and transport of the patient. Hand washing with soaps containing disinfectants like chlorhexidine with alcohol or triclosan containing products, and use of disposable gloves and wearing mask and gown during procedures is effective. No equipment or items should be shared with other patients and instruments to be reused should be thoroughly cleansed

with soap and water and autoclaved before reuse, mattresses used by the patients should be treated with strong sunlight to ensure disinfection and all mattresses should be covered with rexin sheets that can be disinfected.

The use of topical agents to eliminate staphylococcal colonization in high-risk groups, such as patients undergoing hemodialysis or surgery, has been shown to reduce the incidence of subsequent infections. Mupirocin, a topical antistaphylococcal agent that inhibits RNA and protein synthesis, eliminates nasal colonization in carriers and can reduce the incidence of wound infections. Although the development of resistance to mupirocin to date has been limited, prolonged use of the drug has been associated with resistance.

A capsular polysaccharide–protein conjugate antistaphylococcal vaccine has produced improved phagocytosis in vitro and improved survival in experimental models of staphylococcal infection, including endocarditis. Balaban et al demonstrated that immunization with RNAIII-activating protein, an agr-encoded protein involved in regulating the expression of staphylococcal exoproteins, is protective in an experimental model of cutaneous infection.[68] Other potential approaches involve the development of multicomponent vaccines incorporating proteins identified as having a role in the pathogenesis of staphylococcal disease.

At present, prevention of the spread of infection relies on the application of appropriate principles of infection control. These approaches have been effective in reducing the nosocomial spread of staphylococcal infection.

MATERIALS & METHODS

The study was carried out from May 2006 to August 2007 at PSG Hospitals, Coimbatore. Clinical samples such as sputum, blood, pus, CVP tips, tracheal aspirates, urine etc were processed for isolation of Staphylococci. A Gram stain was performed initially, and then the samples were inoculated on two 10% sheep blood agar (one for aerobic and the other for anaerobic), MacConkey and nutrient agar plates. Blood samples sent in brain heart infusion broths were incubated for 18 hours and then sub cultured onto blood, MacConkey and chocolate agar plates. These plates were then incubated at 37^{o} C for 24 to 48 hours. All the suspected colonies were Gram stained and subjected to various biochemical tests to identify and characterize them.

Medical records for the source patients were searched for demographic information, history of prior hospitalization, presence of major conditions, (like Diabetes mellitus) renal dysfunctions, post surgical status, malignancy, organ transplant, and trauma or burn injury. Antibiotic exposure within the preceding year was recorded. Follow up studies were done for 100 patients from whom MRSA was isolated, as to what antibiotic was given and they were followed up till their culture was sterile.

Tests to differentiate members of Micrococcaceae family from the other Gram-positive cocci.

Catalase test[4]

A suspected colony was taken with a wooden rod and transferred onto slide and tube containing 3% hydrogen peroxide and looked for effervescence**.**

Tests for Differentiation of Staphylococci from Micrococci.

Modified Hugh and Leifsons O/f test[4]

The culture under test was inoculated into two tubes of O/f dextrose medium by stabbing down their whole length with a long wire loop. One tube was covered with a layer of sterile liquid paraffin of atleast 2.5 cm deep after inoculating the culture into it and both tubes were incubated at 37°C for five

days. Staphylococci produce acid by fermentation throughout the depth of medium both in the anaerobic tube sealed with paraffin oil and in the aerobic tube. Micrococci fail to produce acid in both the tubes or produce acid in the aerobic tube only.

Susceptibility to Bacitracin [4]

A suspension of bacterium was spread over a plate of Mueller Hinton agar. A disk containing 0.04 units of bacitracin was placed on it and the plates were incubated overnight. Most staphylococci grew upto the disc or showed a zone of inhibition of less than 10mm in diameter

Susceptibility to furazolidine[4]

This test was performed as a disk susceptibility procedure .The organism to be tested was grown in peptone water and matched with 0.5 Mac Farland turbidity standard. The organism was spread on a blood agar plate using a swab. Aseptically a furazolidone disk containing 100 µg was placed in the center of the inoculated area and gently the disk was stamped so that it adheres to the agar surface. The plate was incubated at 35 ºC in an ambient -air incubator for 18-24 hours and then examined**.**

Distinguishing staphylococcus from micrococcus[11]

Property	Staphylococcus	Micrococcus
Anerobic growth	+	_
Carbohydrate utilization	Fermentative	Oxidative
Catalase	+	+
Oxidase	_	+
Sensitivity to Bacitracin	R	S
Sensitivity to furazolidine	S	R

Differentiation of Staphylococcus aureus from other Staphylococci

Coagulase test[4]

Slide coagulase test

This test detects clumping factor, which is present in Staphylococcus aureus. It is absent in most other Staphylococci. The reagent used was rabbit plasma. Rabbit plasma was obtained by centrifuging blood to which 0.1% ethylene diamine tetra acetic acid was added as anticoagulant .The plasma was stored in small portions at 20ºC and the in use plasma was stored at 4ºC and was brought to room temperature before use.

Clumping factor can be detected by standard slide test performed by making a heavy suspension of cells in saline and stirring the mixture to a homogeneous composition and then adding a drop of rabbit plasma .The mixture should be examined for clumping factor within about 10 seconds. Similar suspensions of positive and negative control strains were made and examined.

Tube coagulase test[4]

A 1in 6 dilution of plasma in saline was prepared and 1 ml volume of diluted plasma was placed in small tubes. A colony under test was emulsified in the diluted plasma. Positive and Negative controls were included. The tubes were incubated at 37º C and were examined for clot formation after four hours and if negative, were left at room temperature for the next 12-16 hours and were re-examined for presence of a clot.

Deoxyribonuclease test (DNase test) [4]

DNA agar plates were dried of all the moisture and the plates were divided into sections and strains under test were touched with an inoculating loop and spot inoculation was done. Positive and negative controls were used. The plates were incubated at 37º C for 24 hours and then they were flooded with a few milliliter of 1mol/litre of 3.6% Hydrochloric acid to precipitate unhydrolyzed DNA.After few minutes the plates were examined. Spot cultures that were surrounded by clear cloudy zone comparable in width to zone around the positive control were considered as positive.

Phosphatase test

Phenolphthalein diphosphate agar plates were taken and the strains were inoculated and incubated at 37ºC for 18-24 hours. The next day the growth was exposed to ammonia. The cultures, which turned bright pink, were taken as positive for the production of phosphatase.

Mannitol fermentation

The test organism was inoculated on mannitol salt agar. The medium consisted of 1% mannitol, 7.5%sodium chloride, phenol red and peptones. The plates were incubated at 37 ºC for 18-24 hours and then examined.

Coagulase negative staphylococci

Criteria for Inclusion

Type of specimen	Inclusion Criteria
Urine	$\geq 10^5$ CFU/ml and absence of other recognized bacterial pathogens[a]
Nonurinary	Growth 1+ (few)[b] and single bacterial morphotype of CONS and absence of other recognized bacterial pathogens[a]
	Growth greater than 2+ (moderate) [b] and absence of other recognized bacterial pathogens[a]

[a] Defined as *Staphylococcus aureus*, beta-hemolytic streptococci, and all aerobic gram-negative bacilli.

[b] Defined based on the study[12]

Identification of CONS

Ornithine decarboxylase test[4]

Preparation of medium

Peptone, yeast extract and glucose were dissolved in one litre of distilled water and the ph was adjusted to 6.7. 10ml of bromocresol purple 0.2% solution was added and L ornithine hydrochloride was added to get the final concentration

of 0.5%. Volumes of 3ml was taken in each tubes and sterilized by autoclaving. Once the broth was cool, the test organism was inoculated into it and the tube was overlayered with 5mm of sterile mineral oil. The tubes were incubated at 37ºC for 24-48 hours. Violet coloration of medium indicates production of decarboxylase by the organism.

Urease test[4]

Urea medium was streaked with test culture and they were incubated at 37º C for 18-24 hours and then examined. Urease producing organisms turned the medium dark pink. Phenol red was the indicator used.

Acetoin production test (Voges prauskauer test) [4]

Glucose phosphate broth was inoculated with the test organism and incubated at 37º C for 24 to 48 hours and then 5%alpha naphthol in ethanol and 40%potassium hydroxide were added in the ratio of 3:1 and the broth is shaken vigorously for 30 seconds and aerated and then examined. Reddening of supernatant within 5- 10 minutes was indicative of acid production in the glucose phosphate broth.

Carbohydrate fermentaion

Sugar broth consisted of one of the sugars (1%) (Glucose, trehalose, mannitol, sucrose, maltose, fructose and mannose) 5oogms filtered and added into sterile nutrient broth and andrade's indicator was added into the broths. The test organisms were inoculated into the broths and the broths were incubated at 37 ºC for 18-24 hours.

Novobiocin sensitivity

All the urine samples which grew staphylococci were tested for novobiocin sensitivity. All the strains were lawn cultured on muller hinton agar plates and 5µg novobiocin disc was included in the disc diffusion test for antibiotic sensitivity. *Staphylococcus saprophyticus* shows no zone or zone less than 10 mm in diameter.

Susceptibility to polymyxin B

This test was done on muller hinton agar along with novobiocin susceptibility. A suspension of the organism equivalent to 0.5 Mc Farland turbidity standard was prepared in peptone water and swabbed on a mullerhinton agar plate. A polymyxin B disk (300 units) was aseptically placed in the center of the plate and the plates were incubated at 37º C for 18-24 hours and then examined.

Indentification of staphylococci was done by referring the table I, II & III

Determination of Antibiotic susceptibility of staphylococcus aureus and clinically relevant coagualse negative staphylococci[93]

Antimicrobial susceptibility testing was done on Muller Hinton agar and their susceptibilities to antimicrobials including Oxacillin (1µg), Cefoxitin (30µg), Trimethoprim- Sulfamethoxazole (1.25/23.75µg), Tetracycline (30µg), Linezolid (30µg), Erythromycin (15µg), Clindamycin (2µg), Vancomycin (30µg), Teicoplanin (30µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Penicillin (10units), Amikacin (30µg),Gentamycin (! 0µg), Rifampicin (5µg), Muprocin (5µg), Fusidic acid (10µg) were recorded.

Determination of induced clindamycin resistance (Dtest) [15]

The double disc diffusion test (D test) was performed on all MRSA isolates whose antimicrobial susceptibility pattern were clindamycin susceptible and erythromycin resistant. To detect inducible clindamycin resistance 15µg Erythromycin and 2µg Clindamycin disk were placed 15 -20 mm apart for *Staphylococcus aureus* and 20-26mm for coagulase negative staphylococcus strains. *Staphylococcus aureus* 25923 was used as control for these tests and the plates were incubated at 37 ˚C for 18 to 24 hours .In the double disk diffusion test, if there is inducible Clindamycin resistance, the Erythromycin will diffuse through out the agar, and resistance to Lincosamide will be induced resulting in flattening or blunting of the Lincosamide zone of inhibition adjacent to the Erythromycin disc giving the shape of D to the zone.

Identification of hospital acquired methicillin resistant *Staphylococcus aureus*

MRSA isolates were designated as HAMRSA if the source patient had any of following risk factors: 1) a history of hospitalization, 2) residence in a long term case facility, (eg nursing home), 3) dialysis or surgery within one year prior to date of specimen collection, 4) growth of MRSA after 48 hr or more after admission to a hospital, 5) presence of a permanent indwelling catheter or percutaneous device at the time of culture or prior to this study. If none of above risk factors were present them isolate was considered as CAMRSA.[48]

Detection of Methicillin resistant staphylococcus aureus

All the 610 *Staphylococcus aureus* isolated phenotypically by different biochemical tests, were subjected to susceptibility testing using Oxacillin(1µg), Cefoxitin (30ug)(Himedia) disc diffusion method for detection of methicillin resistance. Out of 610, a total of 250 isolates were subjected to other phenotypic detection methods such as Oxacillin resistance agar screen, detection of MIC of Oxacillin(Himedia) by agar dilution method and using mannitol salt agar for detecting methicillin resistance instead of muller hinton agar.Of the 250 isolates of *Staphylococcus aureus*,100 were selected from those isolates which were sensitive to both Oxacillin and Cefoxitin disk,130 from those isolates which were resistant to Oxacillin and Cefoxitin disk by disk diffusion method and 20 were selected from those which were resistant to Oxacillin but sensitive to Cefoxitin.

Methods for detection of MRSA

Oxacillin Disc diffusion method: [11, 13, 49, 50]

A sterile swab was dipped in Staphylococcus suspension (Mc farland standard 0.5) and was plated on Muller Hinton agar and mannitol salt agar using oxacillin 1µg and plates were incubated at 37^{o} C for 24 hour. The zone of inhibition was documented in millimetres. If the zone diameter is ≤10 mm, then the isolate is resistant to Oxacillin.If the zone diameter is ≥13mm, then the isolate is said to be sensitive

Cefoxitin Disc Diffusion method[11, 13, 49, 50]

A sterile swab was dipped in staphylococcus suspension (Mc farland standard 0.5) and was plated on Muller Hinton agar and mannitol salt agar using Cefoxitin 30µg and plates were incubated at 37° C for 18-24 hour. The zone of inhibition was documented in millimetres. If the zone diameter≥22mm, then the isolate is said to be sensitive, and if the zone diameter is ≤21mm, then the isolate is said to be resistant to Cefoxitin.

Agar screen test: [52] Oxacillin resistance screen agar using 6mg of Oxacillin /litre was used for detection of MRSA. This method was recommended for screening colonies isolated on routine culture media and for confirmation of suspect resistance seen on disc diffusion test. The test organism was grown in peptone water and the density was compared to the density of a 0.5 Mcfarland standard and then the test organism (10µl) was spot inoculated on MH agar (containing 4% sodium chloride and 6mg / l Oxacillin). Plates were then incubated at 35° C for 24 hours. The next day the plates were examined for growth of the organisms. Aniline blue was the dye used in the medium.

Interpretation

Growth of > 1 colony = MRSA

No growth = MSSA

Dilution methods

Agar dilution method[49, 50]

About 18-20 ml of Muller hinton agar medium was prepared in test tubes and allowed to cool in 50º C waterbaths. Serial dilutions of oxacillin powder was prepared in sterile water First 10mg of oxacillin was weighed and diluted in 10ml of sterile water. Seven sterile test testtubes were taken and serial dilutions of stock solutions was done and diluted antibiotic solutions were added to cooled broth in the ratio of 1ml of antibiotic solution to 19ml of the medium. After adding the antibiotic agent in the medium, it was mixed well and poured into Petri dishes. So the dilutions were ranged from 0.5µg/ml to 32µg/ml. A control plate containing the test medium without the antibiotic was prepared for each series of dilution. After the plates were set, they were dried at 37 ºC. Fresh inoculum of an actively growing organism was taken and the turbidity was adjusted to 1 Mac Farland

standard. The dried plates were taken and divided and marked .A platinum loop calibrated to deliver 0.001ml of the inoculum was used to spot inoculate the culture. A quality control organism was also included. Inoculated plates were left undisturbed until the spots of inoculum dried .The plates were then inverted and incubated at 37 ºC for 16-18 hours and then examined for growth.

Molecular detection of MRSA by PCR

Steps

- DNA extraction
- PCR amplification of the extracted DNA
- Gel documentation of the amplified product
- All the isolates were inoculated into semisolid agar medium and stocked.

Bacterial isolates

About 55 phenotypically determined *Staphylococcus aureus* (MRSA and MSSA) were randomly selected from the 250 selected for phenotypic detection of methicillin resistant *Staphylococcus aureus* .Ten from Diabetic patients, five from burns infections, Five from skin infections, ten from surgical site wound infections, six from osteomyelitis cases, fivefrom pneumonia cases, three from UTI, two control strains (MRSA and oxford *Staphylococcus aureus)* nine cases which were resistant by oxacillin disk diffusion but were sensitive by cefoxitin disk diffusion methods were also added in our study.

Materials required for extraction

Chemicals and reagents

- Equilibrated phenols
- Chloroform extra pure
- Isoamyl alcohol
- 10XTBE buffer
- 10 X TAE buffer
- Sodium chloride M grade
- Phosphate buffered saline
- Proteinase K

Fig :
Thermocycler

- Sodium dodecyl sulphate
- Absolute alcohol

DNA Extraction: [69]

Pure DNA was prepared by growing bacteria in 5ml of Brain heart infusion broth for 18 hour. The grown bacteria were centrifuged at 5000 rpm for 10 minutes and supernatant discarded. The sediments were resuspended in lysis buffer and incubated at 37^{o} C for 1 hour. Equal amount of phenol: chloroform mixture was added and vortexed for 30 seconds. The tubes were centrifuged at 10,000 rpm at 5min. at room temperature and the top aqueous layer was collected into Fresh sterile tube. To this 60µl of 3M Nacl and 3 volumes of cold absolute ethanol was added and the tubes were kept at -20^{o} C overnight for precipitation. The tubes were centrifuged at 10000 rpm for 30 minutes at 4^{o} C.

The supernatant was discarded and 200µl of 70% Ethanol was added to pellet and centrifuge again at 10,000 for 15 min. The supernatant was discarded and the pellet was air-dried. The pellet was dissolved in 0.5ml of T10E Buffer

PCR reaction: [69]

PCR mixture consist of

- Mec A Primer

  Forward and reverse primers of MecA

  Forward primer -5'AAATCAGATGGTAAAGGTTGGC3'

  Reverse primer-5'AGTTCTGCAGTACCGGATTTGC3'
- Fem A Primer

  Forward primer-5'AAAAAAGCACATAACAAGCG3'

  Reverse primer-5'GATAAAGAAGAAACGAGCAG3'
- d NTP
- Tag buffer
- Tag polymerase
- 2 µl of extracted DNA template

By using 2.5µl of template DNA and 2 µl each of both primers (forward and reverse) 2ul each, dNTP-0.8ul, Tag Buffer -2µl and tag polymerase 0.2ul were

added and DNA amplification was carried out for forty cycles in 20ul of reaction mixture as follows.

1. Denaturation at 95^{o}C for 45 seconds
2. Annealing at 55^{o} C for 45 seconds
3. Extension at 72^{o} C for 1 minute
4. A final extension at 72^{o} C for 5 minutes

The amplified product is now ready for analysis

Analysis of PCR product: [69]

The PCR product was analyzed in 1.5% agarose gel prepared using TAE buffer and 0.5ug / ml ethidium bromide. The molten agarose gel was cast in the electrophoresis tank with combs. Once the gel solidified, the comb was removed and TAE buffer was added to completely immerse the gel. 10µl of amplified PCR product was mixed with 2ul of 0.1 bromophenol blue, (tracking dye) and were loaded into the wells. A DNA ladder of 100 base pairs with tracking dye and amplified product of methicillin resistant staphylococcus aureus control strain and oxford stain of staphylococcus aureus were loaded in each run. After 1 hour the gel was removed and documented in gel documentation system. The MecA gene was recognized by presence of 533 bp band and FemA gene was by the presence 132 bp band.

Statistical methods used for analysis of observation and results.

Chi square test were used to compare proportions. Percentages were calculated where necessary.Sensitivity and Specificity were used to test the performance of detection methods of MRSA.

RESULTS

Out of the 24321 samples sent for cultures during the study period, 5919 samples were culture positive and of these 651(10.9%) isolates were staphylococci. Of the 651 *Staphylococcus* species isolated, 610 were identified as *Staphylococcus aureus* (Fig.1 & Fig.2) and 41 as clinically relevant CONS. Among the 610 *Staphylococcus aureus*, 208 were identified as MRSA (34.1%) based on cefoxitin resistance and 402 as MSSA based on cefoxitin susceptibility. (Table No.1) Of the total MRSA's, 82% were hospital acquired and 18% were community acquired.

Table No.1Prevalence of Staphylococcus aureus amongst all clinical isolates in PSG Hospitals

Total No. of clinical samples during the study period	24321
Total No. of samples which were culture positive	5919 (24.36%)
Total No. of Staphylococci amongst all isolates	651 (10.9%)
Total No. of SA (% among all Staphylococci)	610 (93.7%)
Total No. of MSSA (% among all S.aureus)	402 (65.9%)
Total No. of MRSA (% among all S.aureus)	208 (34.1%)
Total No. of clinically relevant CONS (% among all Staphylococci)	41 (6.3%)

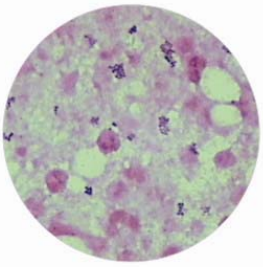
Distribution of staphylococcus aureus amongst different samples

Staphylococcus aureus was the predominant single isolate in pus, catheter tips and synovial fluid samples. (Table No.2) Maximum number of SA was isolated from pus (63%), followed by urine samples (14%), catheter tip(8%) and blood (7%).(Fig.3) Staphylococcus aureus were methicillin sensitive in 82% of isolates in blood, 67% in pus and 56% in urine, whereas from catheter tips, 85 % were methicillin resistant (Table No.2)

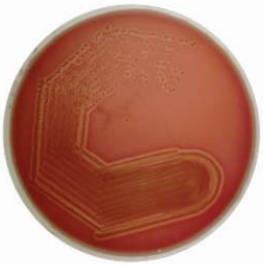
Table No.2 Prevalence of S.aureus among isolates in various samples

Sample	MRSA	MSSA	Total SA	Others organisms	Total
Pus	128(32.48%)	267(67.5%)	395(20.8%)	1503(79.2%)	1898
Catheter tip	17(85%)	3(15%)	20(11.83%)	149(88.1%)	169
Urine	30(44.11%)	38(55.88%)	68(3.4%)	1894(96.6%)	1962
Blood	15(18.07%)	68(81.92%)	83(17.44%)	476(82.56%)	559
BAL	2(40%)	3(60%)	5(11.1%)	40(88.9%)	45
Sputum	6(46.15%)	7(53.84%)	13(2.24%)	565(97.7%)	578
Tracheal aspirate	7(46.7%)	8(53.3%)	15(2.9%)	498(97.26%)	512
Synovial Fluid	0(0%)	2(100%)	2(50%)	2(50%)	4
Throat swab	0(0%0	1(100%)	1(3.22%)	30(96.77)	31
Ear swab	3(37.5%)	5(62.5%)	8(4.9%)	153	161
Total	208(34.09%)	402(65.9%)	610	5310	5919

Fig :
Morphological Characteristics of Staphylococcus aureus



Puscells & Gram postive Cocci in Clusters



Beta Hemolytic Colonies on Blood agar

Small Lactose Fermenting Colonies on Macconkey agar

Golden Yellow Pigment on Milk agar

Cream Pigmentation on Nutrient agar

Fig :
Identification of Staphylococcus aureus

Mannitol Fermenting Yellow Colonies

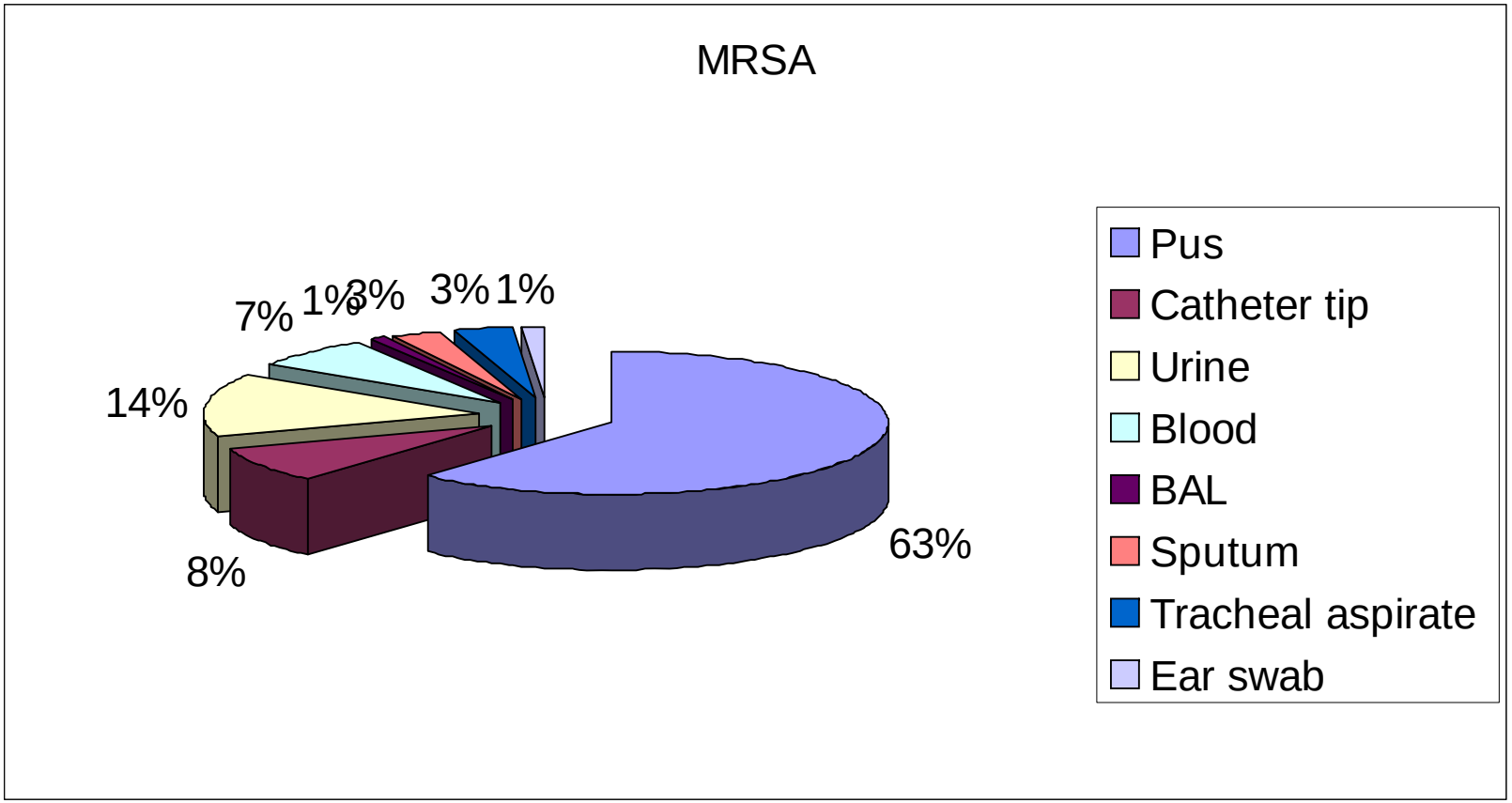
Phosphatase Test
Pink Colour Colonies produced by Staphylococcus aureus

DNA ase Test
Above --Test
Below -- Control

Tube Coagulase Test
Right : Positve for Tube Coagulase
Left : Negative for Tube Coagulase

Fermentation of Mannitol
Right : Staphylococcus aureus
Left : Staphylococcus epidermidis

Fig .3 : Distribution of *MRSA* among different samples



Age and sex wise distribution of Staphylococcus aureus among patients:

Patients above 40 years were significantly more affected with MRSA than other age groups (p=.0027). Only 1.1% of isolates of Staphylococcus aureus were from neonates. (Table 2) The percentage of males affected with MRSA was 34.22% and the females affected were 33.89%. (Table 3) There was no significant difference between MRSA occurrence among males and females.

Table No 3: Age wise distribution of Staphylococcus aureus

Age	MRSA No (%)	MSSA No (%)	Total (610)
Neonates	2 (0.9%)	5 /1.24%	7 (1.1%)
< 14years	15 /7.2%	67 /16.6%	82 (13.4%)
14-40 years	72/ 34.6%	108 /26.8%	180 (29.5%)
> 40 years	119 /57%	222/55.2%	341 (56%)

Table No.4: Sex wise distribution of Staphylococcus aureus

Sex	MRSA	MSSA	Total (610)
Male	128(34.22%)	246(65.8%)	374 (61%)
Female	80(33.89)	156(66.11%)	236 (39%)

Frequency of MRSA in various infections as compared to other organisms

Among the ICU cases, 17% were contributed by MRSA, rest were contributed mainly by Pseudomonas aeroginosa, Acinetobacter baumani, Klebsiella pneumonia, Candida and Aspergillus sp. Among all patients with infected burns, MRSA was isolated in 16% cases. In most infections MSSA was isolated more than MRSA, marginally, except in burns, where MRSA was present in more percentage of cases.(Table 5)

Table No 5: Comparison of frequency of MRSA versus other bacteria in various infections:

Infections	MSSA (%)	MRSA (%)	SA (%)	Other bacteria (%)
Burns	4.2	16	20.2	79.8
Surgical site infection	10.6	3.2	13.8	86
Diabetic wounds	9	4	13	87
ICU	20	17	37	63
VAP	8	9	17	83
Neonatal infections	6	2	8%	92%

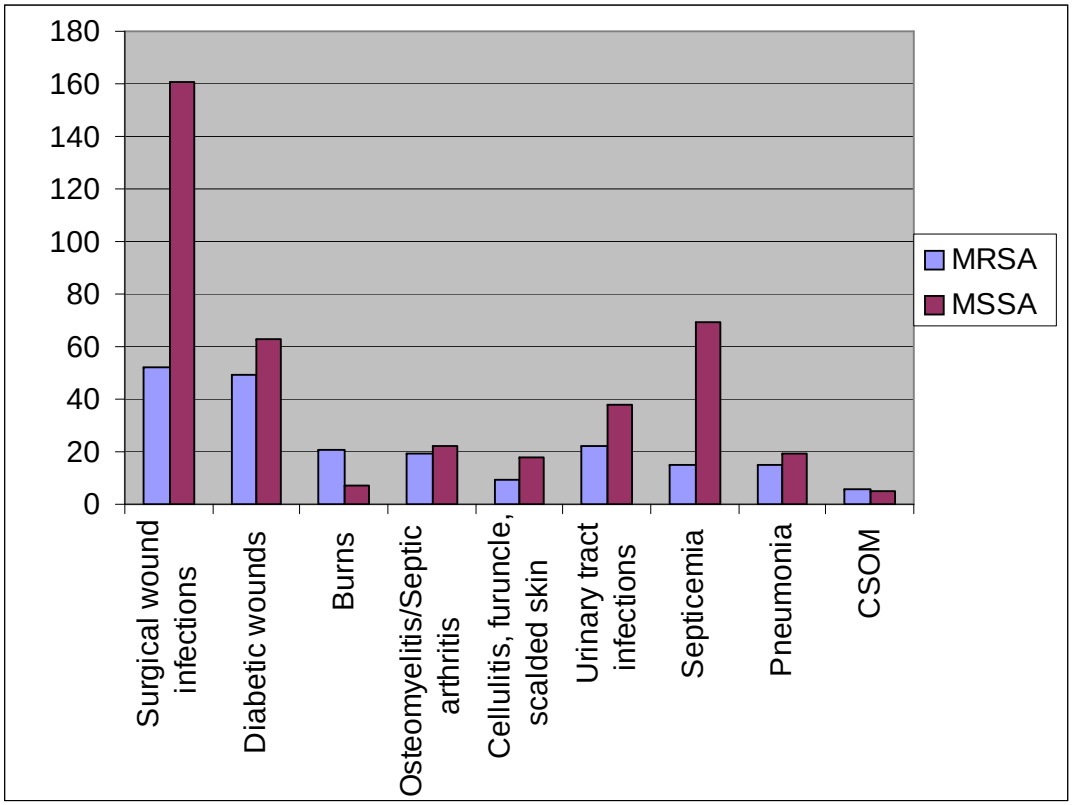
Distribution of Staphylococcus aureus among various infections

Maximum number of Staphylococcus aureus was isolated from surgical site infections (34.9%) and diabetic wounds (18.36%). Septicaemias constituted 13.8% of all staphylococcus aureus infections. Staphylococcal skin infections such as cellulitis, furuncle and Staphylococcal scalded skin syndrome constituted 4.4% of infections. MSSA was predominant among most infections except in burns, where 75% were MRSA. (Table 6, Fig 4)

Table No.6: Distribution of Staphylococcus aureus among different infections

Diagnosis	MRSA	MSSA	Total SA
Surgical wound infections	52(24.41%)	161(75.58%)	213 (34.9%)
Diabetic wounds	49(43.75%)	63(56.25%)	112 (18.36%)
Burns	21(75%)	7(25%)	28 (4.6%)
Osteomyelitis/Septic arthritis	19(46.34%)	22(53.65%)	41 (6.7%)
Cellulitis,furuncle, scalded skin syndrome	9(33%)	18(66%)	27 (4.4%)
Urinary tract infections	22(37%)	38(63%)	60 (9.8%)
Septicemia	15(17.9%)	69(82.1%)	84 (13.8%)
Pneumonia	15(44%)	19(56%)	34 (5.8%)
CSOM	6(55%)	5(45%)	11(1.8%)
Total	208(34.1%)	402(65.9%)	610

Fig.4: Distribution of Staphylococcus aureus among different infections (in numbers)



Distribution of MRSA among various infections

Total number of MRSA isolated was 208. Most Number of cases of MRSA was isolated from surgical site infections(25%) followed by diabetic patients (23.6%), UTI (11%), burns cases (10%),Osteomyelitis(9%) and skin infections (4.3%), Pneumonia cases(7%), CSOM(3%) and Septicemia (7%)(Fig.5)

Staphylococcal skin infections

Only 27 cases of classical Staphylococcal skin infections were seen in this study among the 610 Staphylococci isolated. Predominant Staphylococcal skin infections were folliculitis and cellulitis. Majority (66%) were Methicillin Sensitive. All the MRSA here were community acquired except the lone Staphylococcal Scalded Skin syndrome which was hospital acquired. This case of toxic epidermolysis grew MSSA initially, but subsequently after prolonged hospitalization, MRSA became the predominant isolate. (Table 7, fig 6,7)

Table 7: Distribution of Staphylococcus aureus among different skin infections (n=27)

Skin infections	MSSA	MRSA	Total
Carbuncle	3	2	5
Furuncle	2	1	3
Folliculitis	8	2	10
Cellulitis	5	3	8
Staphylococcal scalded skin syndrome	0	1	1
Total	18(66%)	9(33%)	27

Fig.5: Distribution of MRSA among different infections

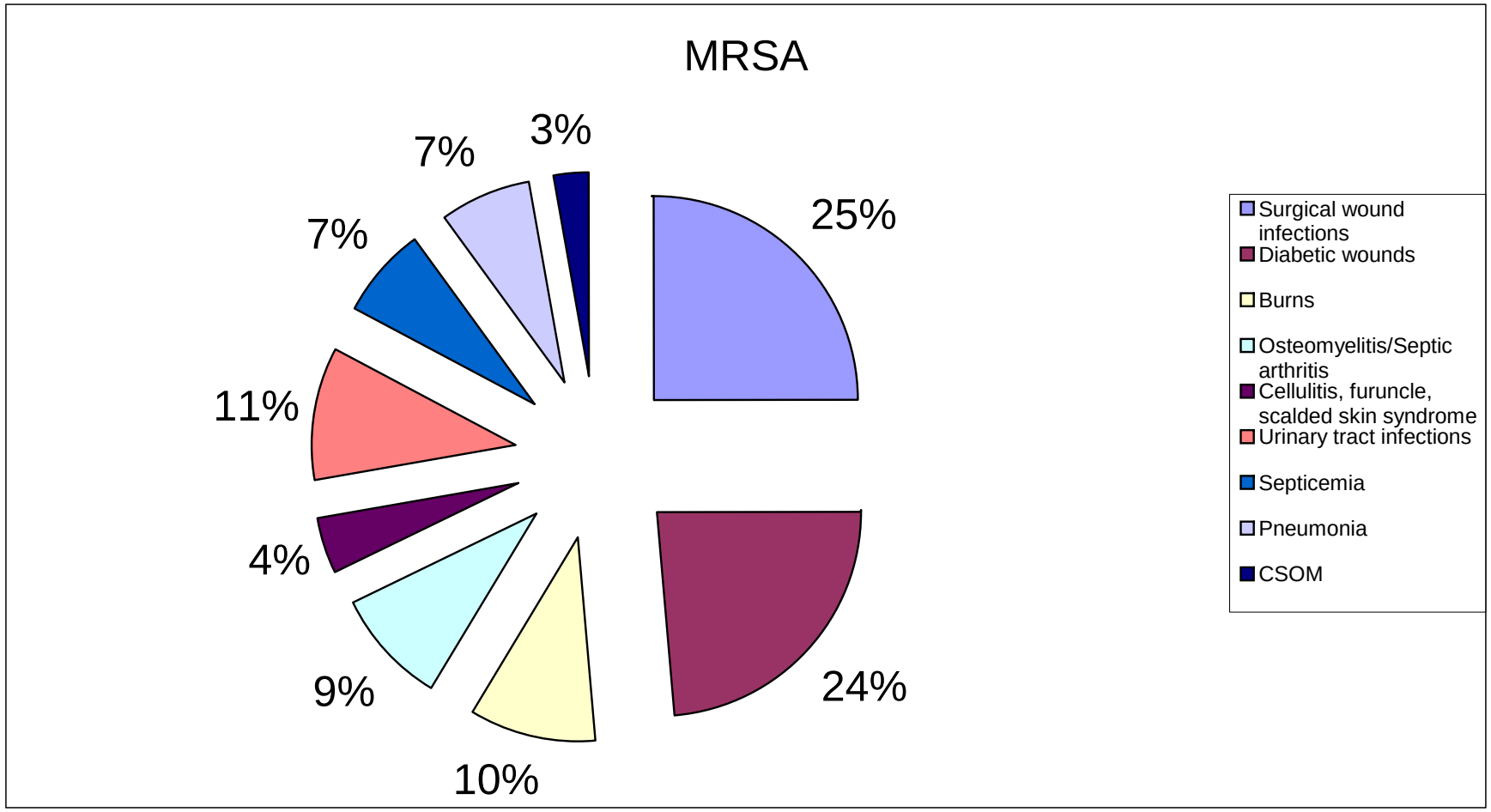


Fig. 6: Distribution of Skin infections (in numbers)

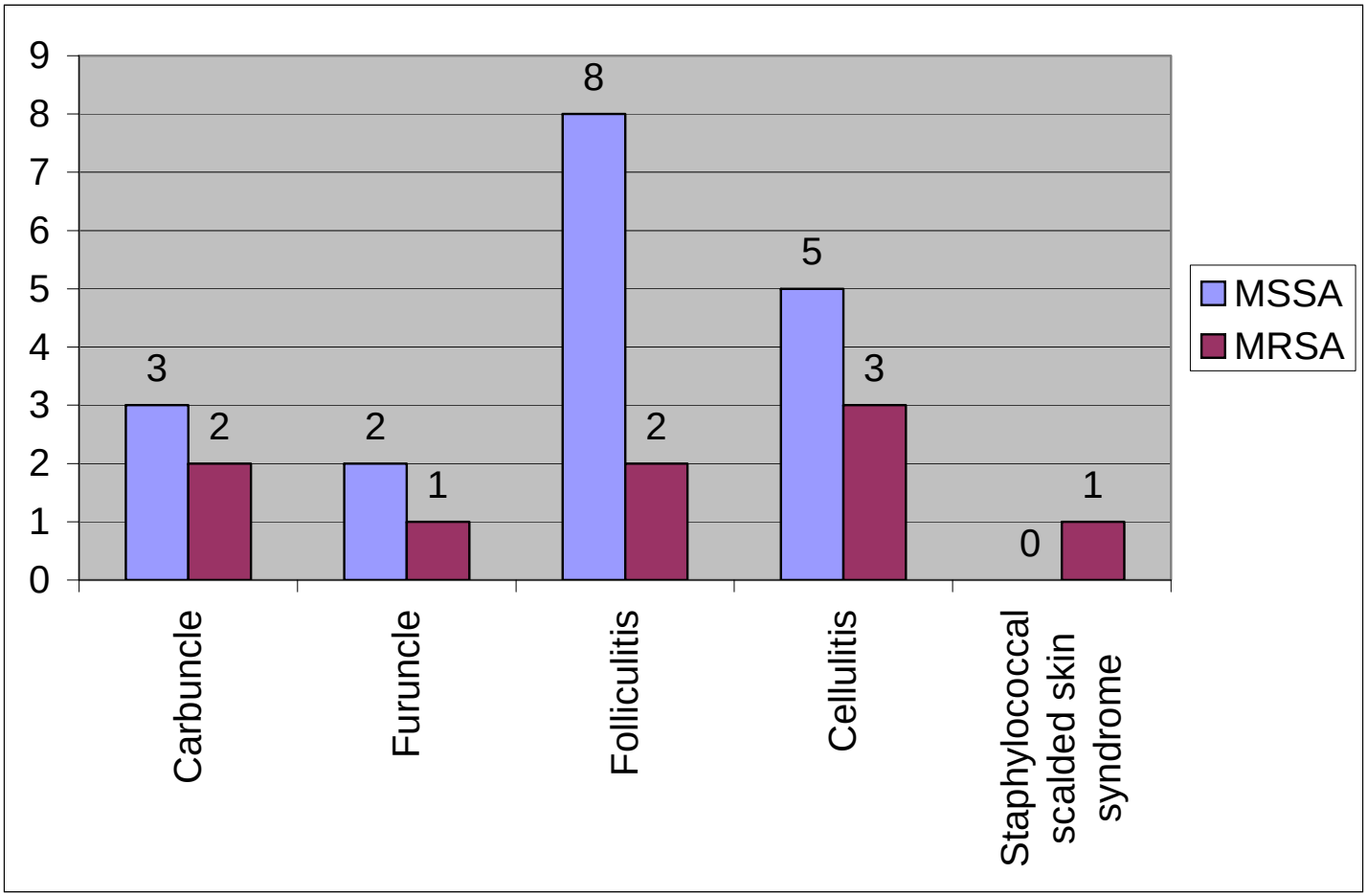


Fig :
Classical Infection Caused By Staphylococcus Aureus In Community

Furuncle

Carbuncle

Staphylococcal Scalded Skin Syndrome

Antibiogram of *Staphylococcus aureus*

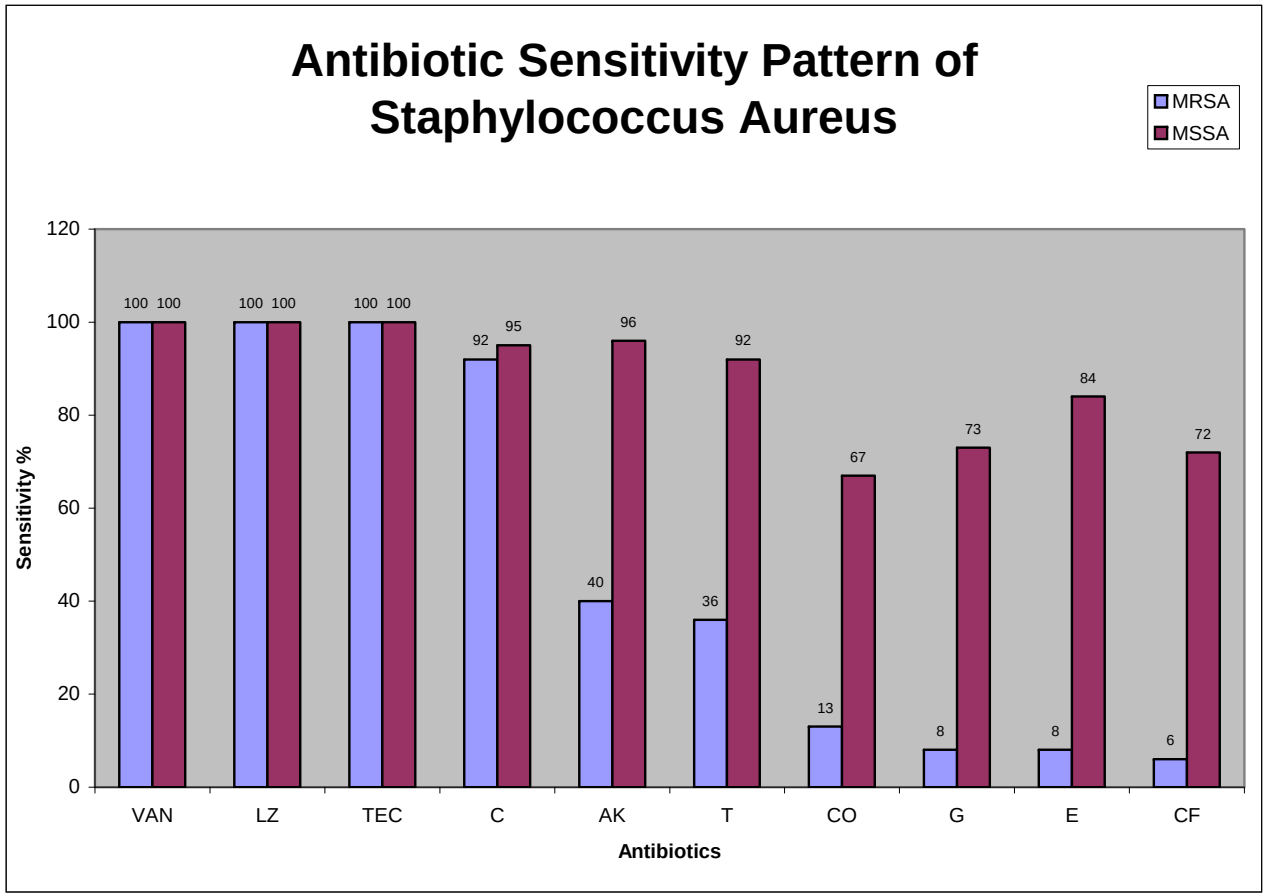
The antibiotic susceptibility pattern of all the isolates of *Staphylococcus aureus* are depicted in the bar diagram. All the isolates were sensitive to Vancomycin, Linezolid and Teicoplanin. Penicillin sensitivity was found to be 22% among MSSA isolates. Most MSSA isolates were sensitive to Gentamicin (73%), Erythromycin (84%), Ciprofloxacin (72%), Tetracycline (92%) and Amikacin (96%) A significant difference was demonstrated between the sensitivity of MRSA and MSSA to Tetracycline, Cotrimoxazole, Gentamicin, Amikacin, Erythromycin and Ciprofloxacin. (Table No. 9, fig 8) Such a difference was not observed with Chloramphenicol. Induced resistance to Clindamycin was found in39.4% of MRSA isolates, constitutive resistance was found in 33.7%,D test was negative in 18.9% and 8% were sensitive to both Clindamycin and Erythromycin . (Fig .9)

Table No.9: Antibiotic Sensitivity pattern of *Staphylococcus aureus*

Antibiotics	MSSA (% S)	MRSA (% S)	P value (Significance of difference in sensitivity)
Penicillin	22	0	<0.05(S)
Tetracycline	92	36	<0.05(S)
Amikacin	96	40	<0.05(S)
Erythromycin	84	8	<0.05(S)
Cotrimoxazole	67	13	<0.05(S)
Gentamycin	73	8	<0.05(S)
Ciprofloxacin	72	6	<0.05(S)
Chloramphenicol	95	92	>0.05(NS)
Vancomycin	100	100	>0.05(NS)
Linezolid	100	100	>0.05(NS)
Teicoplanin	100	100	>0.05(NS)

Note : S – Significant, NS- Not Significant

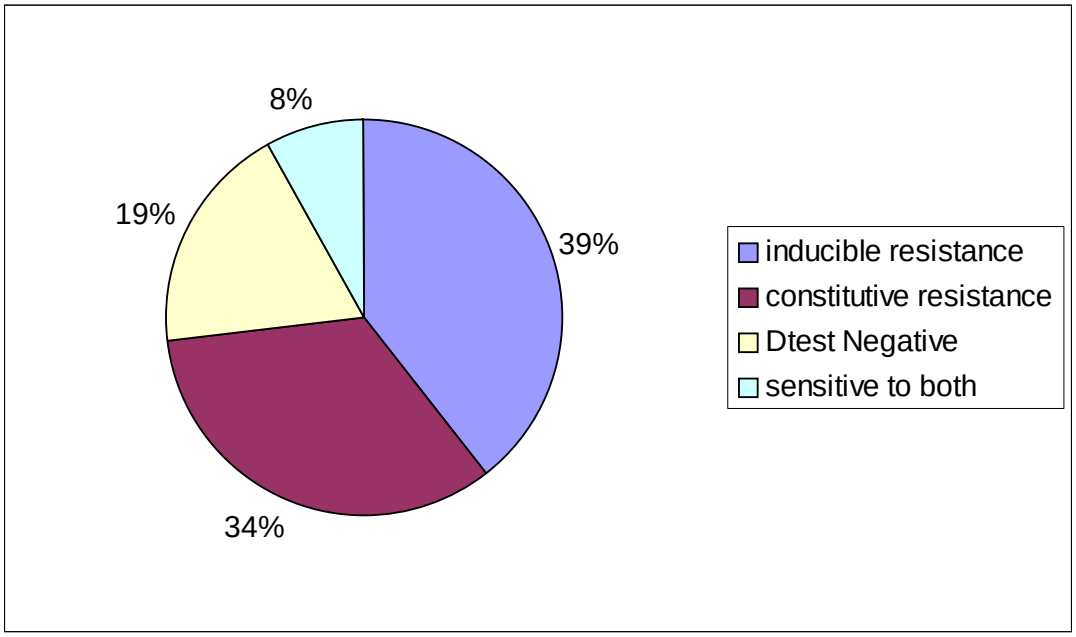
Fig 8: Antibiotic Sensitivity pattern of *Staphylococcus aureus*



Detection of inducible resistance to clindamycin in MRSA

The D test was positive in 39% of all MRSA isolates, indicating that they exhibited inducible resistance. Only 19% of MRSA showed true sensitivity to Clindamycin as the D test was negative in these isolates. Susceptibility to both Erythromycin and Clindamycin was seen in 8% of isolates (fig.9).

Fig 9: Susceptibility of MRSA isolates to Clindamycin and Erythromycin



Susceptibility pattern of CAMRSA as compared to that of HAMRSA

A significant difference was demonstrated between the sensitivity of CA MRSA and HAMRSA to ciprofloxacin. (p=0.008). More numbers of CAMRSA were found to be sensitive to antibiotics like Amikacin, Erythromycin, Cotrimoxazole, Tetracycline and Chloramphenicol than HAMRSA, but this fact was not statistically significant.

Table 9: Antibiogram of CAMRSA as compared to that of HAMRSA

Antibiotics	CAMRSA (%) Sensitive	HAMRSA (%) Sensitive	P value (significance of difference in sensitivity)
Ciprofloxacin	16	0	0.008(S)
Erythromycin	3.2	0	0.3368
Amikacin	41.93	24	0.128
Cotrimoxazole	20	7.4	0.1560
Tetracycline	20.8	11.29	0.3024
Chloramphenicol	10.34	7.4	0.690

Note: S-Significant

Coagulase negative staphylococci

All the 41 CONS isolated were from patients with history of indwelling catheters/ on artificial valves or from patients who were on ventilators and other support systems. *Staphylococcus epidermidis* 24(58.5%) was found to be the predominant species among all CONS, followed by *Staphylococcus* haemolyticus 6 (14.6%), *Staphylococcus* cohini 3 (9.7%), *Staphylococcus h*ominis 4(7%), *Staphylococcus s*aprophyticus 3(7%) and *Staphylococcus s*imulans 1(2.4%). Distribution of Clinically relevant CONS among various samples and the their percentage is depicted in table No10 and fig.10.

Table 10: Species Distribution of clinically relevant CONS

Species	Pus	Blood	Urine	Total
S. epidermidis	20	4	0	24
S. haemolyticus	3	3	0	6
S. saprophyticus	0	0	3	3
S.cohini	0	4	0	4
S. hominis	2	1	0	3
S. simulans	1	0	0	1
Total	26	12	3	41

Antibiotic sensitivity pattern of CONS

Majority of clinically relevant CONS identified were resistant to cloxacillin. All the strains were sensitive to Vancomycin, Linezolid and Teicoplanin. MSCONS were more sensitive as compared to MRCONS. (Table 10,fig 11)

Table No. 11: camparison of antibiotic susceptibility pattern of mscons and mrcons

Antibiotics	MSCONS (% S)	MRCONS (% S)
Vancomycin	100	100
Linezolid	100	100
Teicoplanin	100	100
Chloramphenicol	96	91
Ciprofloxacin	78	22
Cotrimoxazole	55	20
Amikacin	97	69
Gentamycin	72	17
Erythromycin	65	17
Tetracycline	79	75

Fig.10 Distribution of various species of CONS

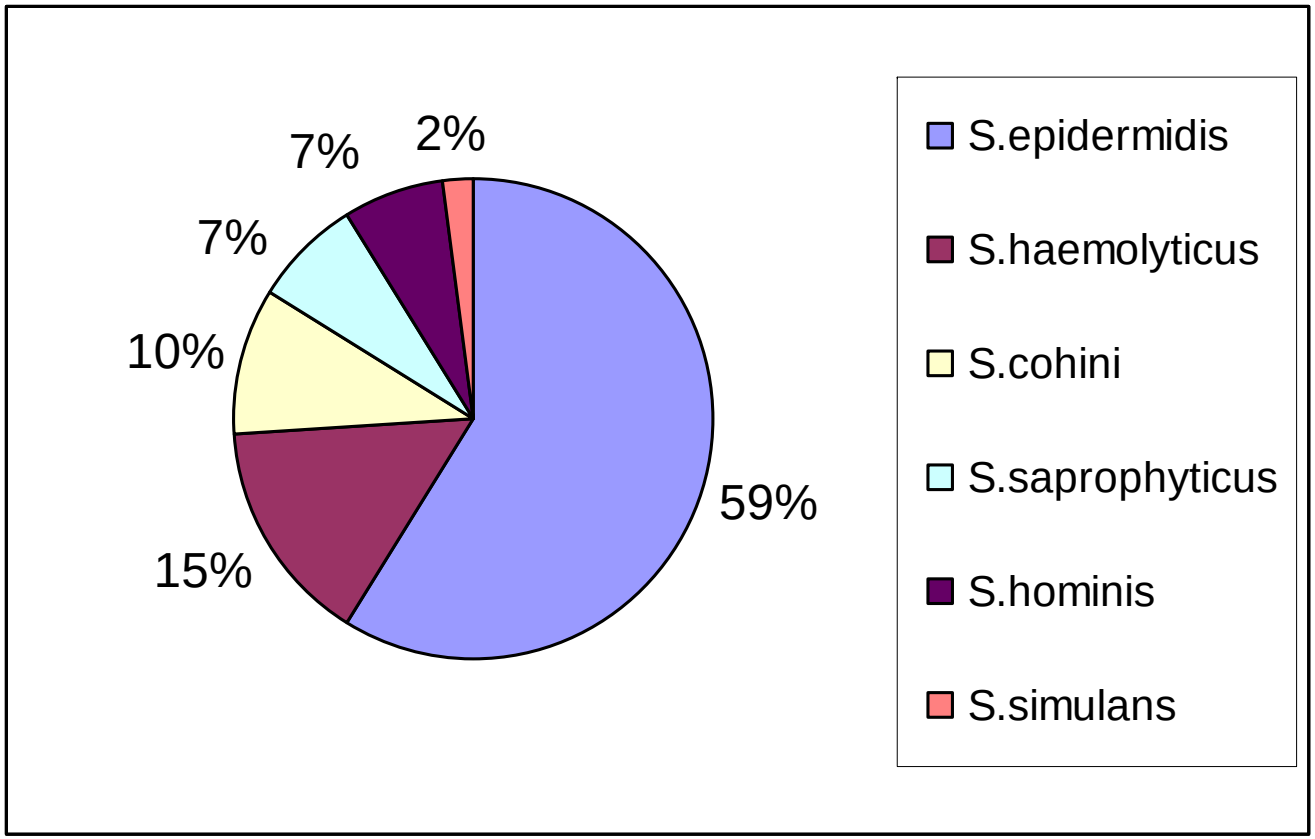
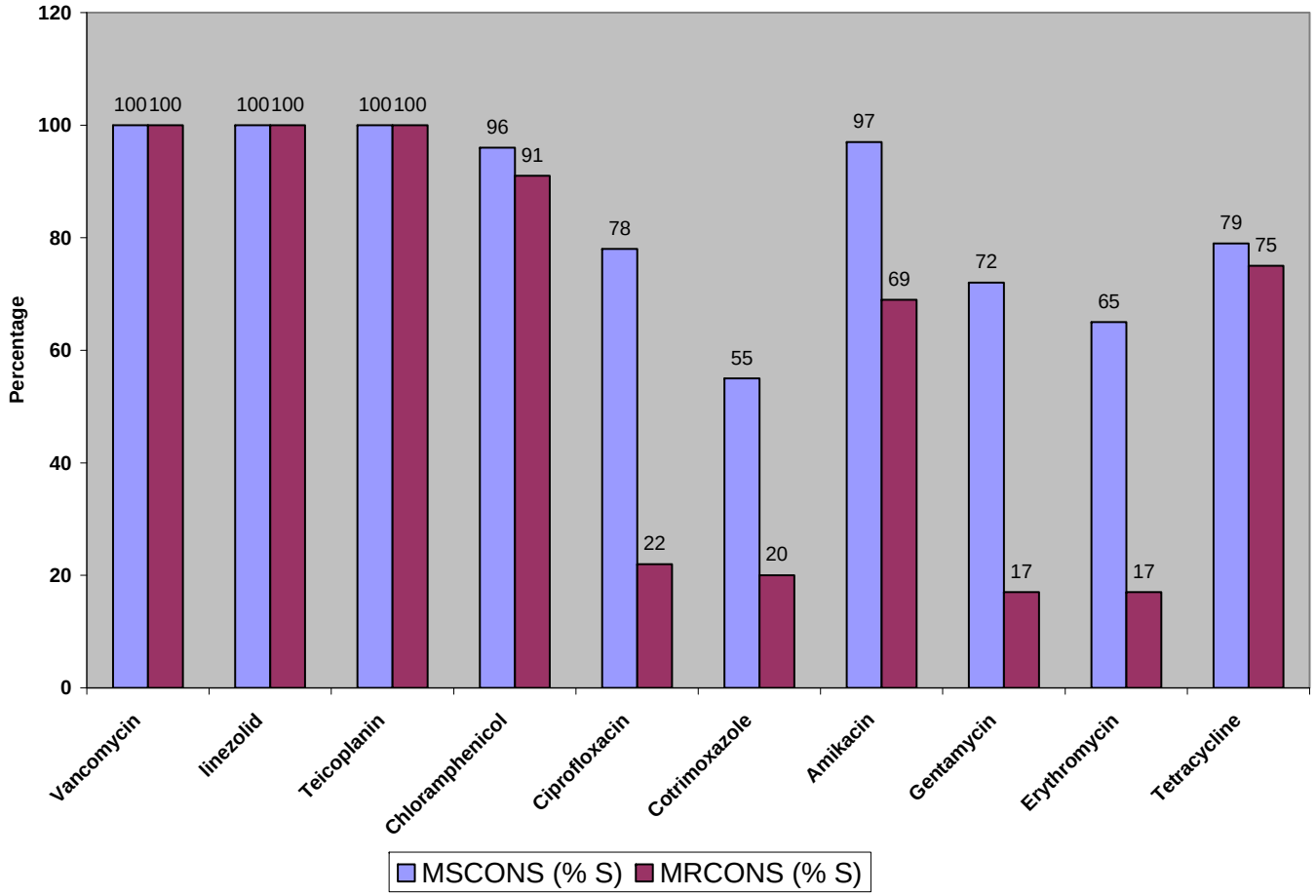


Fig.11 :Antibiogram of Coagulase Negative *Staphylococci*



Detection of MRSA by different phenotypic methods

Out of 610 Staphylococcus aureus isolates, 382 (62.6%) were identified as MSSA based on cefoxitin and oxacillin susceptibility. A total of 208 (34.09%) isolates were identified as MRSA based on cefoxitin and oxacillin resistance. The remaining 20(3.2%) isolates were resistant to oxacillin but sensitive to cefoxitin. These isolates showing a discrepant result were subjected to further phenotypic and genotypic detection methods and were confirmed as MSSA**.** When cefoxitin and oxacillin disc diffusion tests were compared with MIC by agar dilutions tests, it was found that, those strains which were resistant to oxacillin and sensitive to cefoxitin by disk diffusion method had an MIC of 4-8 µg/ml, while the isolates which were sensitive to both oxacillin and cefoxitin had an mic of 0.5-2 µg/ml (<2µg/ml=MSSA). Those isolates that were resistant to both cefoxitin and oxacillin, had an mic of > 16µg/ml. Phenotypic detection of MRSA is shown in fig 12 & fig.13.

Detection of Methicillin resistance by molecular method and its comparison with detection by phenotypic methods.

Of the 55 isolates tested for detection of methicillin resistance by phenotypic and molecular methods, 44 were methicillin resistant by oxacillin disc diffusion and mannitol salt agar methods, whereas only 35 were resistant by cefoxitin disc diffusion and PCR methods. By Oxacillin disk diffusion method, the Sensitivity for identification of MRSA was 100% and Specificity was only 56%. Therefore the percentage of false positives was 45%, and that of false negatives was 0%. By Cefoxitin disk diffusion, sensitivity and specificity was 100% and percentage of false negatives and false positives were 0%. Using Mannitol salt agar instead of muller hinton agar and with the kirby bauer disk diffusion method, sensitivity and specificity were 100 amd 56% similiar to oxacillin disk diffusion method using muller hinton agar. A detailed result of detection by different methods is as follows. Genotypic detection of MRSA is shown in Table 13, fig.14.

Fig :
Detection of MRSA by different Phenotypic Methods

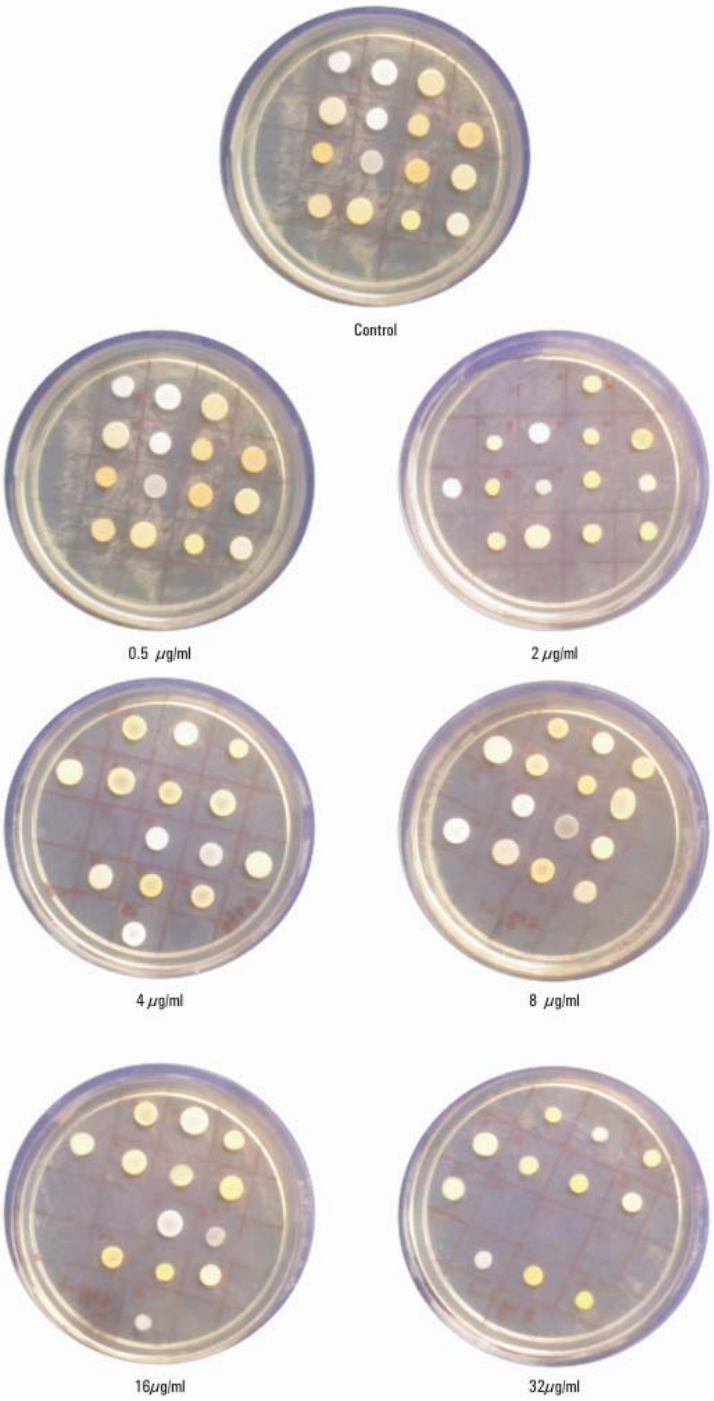
MRSA
Oxacillin Resistant
Cefoxitin Resisitant

Oxacillin Resistant Screen agar

Growth = MRSA
No Growth = MSSA

D test Positive
(Induced Clindamycin Resistance)

Fig :
Determination Of MIC by Agar Dilution



Control

0.5 μg/ml

2 μg/ml

4 μg/ml

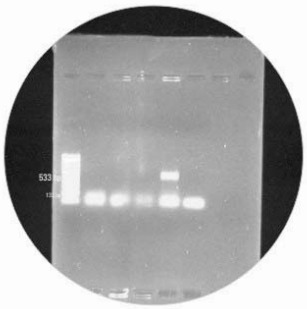
8 μg/ml

16μg/ml

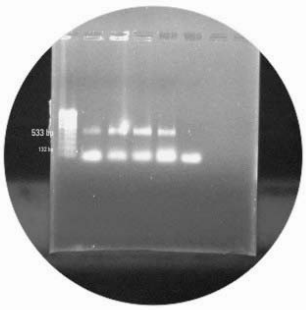
32μg/ml

Fig :
Genotypic Detection of MRSA

Gel Picture of the Extracted DNA



Lane 1 -- Ladder
Lane 2 -- MSSA
Lane 3 -- MSSA
Lane 4 -- MSSA
Lane 5 -- MRSA
Lane 6 -- MSSA



Lane 1 -- Ladder
Lane 2 -- MRSA
Lane 3 -- MRSA
Lane 4 -- MRSA
Lane 5 -- MRSA
Lane 6 -- MSSA

Table 13: Comparison of detection of MRSA by different methods (n=55)

Method of detection	MRSA	MSSA	Sensitivity (%)	Specificity (%)	Predictive value of positive test(%)	Predictive value of negative test(%)
Oxacillin disk diffusion method	44	11	100	56	79.5	100
Cefoxitin disk diffusion method	35	20	100	100	100	100
Mannitol salt agar (using 1 mg disk of oxacillin)	44	11	100	56	79.5	100
Oxacillin resistance screen agar	40	15	97.5	75	87.5	100
MIC of oxacillin using agar dilution	37	18	100	90	94.6	100
PCR- Detection of MecA and Fem A gene	35	20	100	100	100	100

By determination of MIC by agar dilution, all the strains that were MSSA by other methods had an MIC of <=2µg/ml and they were negative for mecA gene by PCR. All the strains that were MRSA by cefoxitin disk diffusion method had a MIC of >32 µg/ml and they were positive for mecA gene. All the isolates that had an MIC of 4- 8 µg/ml were MRSA by agar dilution, but they were negative for mecA gene. By agar dilution method, sensitivity was 100% and specificity was 90%)

Methods of detection of MRSA by oxacillin disk diffusion, Cefoxitin disk diffusion and PCR, all gave a sensitivity of 100% but only the Cefoxitin disk diffusion and PCR methods were 100% specific. Oxacillin disk diffusion was false positive in 45% of cases.

Follow up of patients from whom MRSA was isolated

Out of the 208 cases from whom MRSA was isolated, 100 patients were followed up,of this 66 patients were treated with Linezolid, 12 with Amikacin, 10 with Ciprofloxacin and 9 with Chloramphenicol. Among the 66 treated with Linezolid, in two patients alone, due to delay in wound healing, the patient was subsequently treated with Vancomycin. Doxycycline was given to two patients and in one patient alone Doxycycline was given in combination with Vancomycin. In all patients, the infections healed without further complication.

DISCUSSION

For the past 50 years, *Staphylococcus aureus* has been a dynamic human pathogen that has gained the deepest respects of clinicians. It's incredible success in causing disease involves characteristics associated with host susceptibility (age, chronic disease, surgery, presence of invasive devices or impaired immunity), the specific strain (genetics, ease of colonization, virulence and antibiotic resistance), epidemiology (carriage, transmission) and breaches in infection control measures.[70] In this study Staphylococci constituted 10% of all isolates during the study period and among these, 93.7% were Staphylococcus aureus.

Since the first report of healthcare associated methicillin resistant *Staphylococcus aureus* infection in US at the Boston city hospital in 1961, [6] MRSA has become widespread all over the world. In our hospital too, a prevalence of 34% is seen. Its incidence in other places in India ranges from 30-70%. [1, 8,57,72,73] Similar pattern has been seen worldwide.[71]

There was a significant difference between different age groups in prevalence of MRSA. Elderly patients were significantly ($p = 0.0027$) more prone for infections with MRSA as compared to children and young adults. This might be because elderly are more immunocompromised and are affected by debilitating illnesses like diabetes, more than the young people. Staphylococci were an important cause of infection among the neonates' upto the 1980s. However, nowadays an overall predominance of Gram negative bacteria is noticed.[103,104] In our study also, 92% of the isolates in neonates were Gram negative and only 8% were caused by *Staphylococcus aureus.* Almost half of these were MRSA.

Most cases of MRSA in our hospital were contributed by surgical site wound infection (25%). There are many studies in support of the same in India.[28, 29] and abroad[61] Extensive use of indwelling devices and breaches in infection control measures are cited as important reasons for the predominance of Staphylococcus in these sites. [29]

Methicillin-resistant *Staphylococcus aureus* (MRSA) have also been isolated with increasing frequency from wound and skin infections that commonly affect the lower

extremities of patients with diabetes.[25] In our study MRSA was isolated from 24% of diabetic patients. Antibiotic resistance is reportedly a growing problem in diabetic foot infections.

Burns provide a suitable site for bacterial multiplication. *Staphylococcus aureus* is the most common bacteria isolated from these patients. A direct correlation between the length of stay of burn patients and the risk of becoming infected with MRSA was seen in this study, as was in other studies also.[43] Although in our study only 16% of MRSA was seen among burns cases, a slightly higher rate was reported in other studies.(24% - 45%) [30,31,34,74] In fact among all infections caused by *Staphylococcus aureus*, burns infection is the only one where MRSA (16%) is isolated more than MSSA(4%).

There were only 27 cases of classical *Staphylococcus aureus* skin infections seen in this study, and the predominant infections were folliculitis and cellulitis. Majority were methicillin sensitive.

Pneumonia due to MRSA was contributed by 7.21% as compared to 17% in another study. [35]

In our ICU, 17% of infections were caused by MRSA. There are studies quoting 30% of ICU infections being contributed by *Staphylococcus aureus*, out of which 60% were identified as MRSA. [97]

The incidence of hospital acquired infections among all MRSA's was about 82% in our study, and the patients were mostly diabetics (24%) or had surgical wound infections(25%). The incidence of community based MRSA in our study was about 18% and most cases were contributed by skin infections such as cellulitis and folliculitis, which is similar to other studies.[43] The pattern of incidence of community acquired MRSA by different studies ranges from 7-12.7% in India[1] and 12-44% world wide[15,64,65,91].

The antibiotic sensitivity results showed all MRSA isolates to be significantly more multidrug resistant as compared to MSSA isolates.($p=<0.001$) In our study only about 40% of MRSA were sensitive to Amikacin and Tetracycline, and and less than

15% were sensitive to Cotrimoxazole, Gentamycin, Erythromycin and Ciprofloxacin. Chloramphenicol alone showed 92% sensitivity as was MSSA too (95%). Excepting some, [59] most studies report similar high resistance to Ciprofloxacin, Gentamycin, Erythromycin and Cotrimoxazole.[60,61,75,93]

On the contrary, our MSSA isolates showed more than 65% susceptibility to gentamycin, amikacin, erythromycin, cotrimoxaxole and ciprofloxacin. All our isolates were sensitive to vancomycin, linezolid and teicoplanin but there are a few studies reporting vancomycin intermediate susceptible(VISA) and resistant strains(VRSA).[57,76,101,102] A significant proportion of our isolates of MSSA (22%) were susceptible to penicillin as compared to only <5% isolates being susceptible in other studies. [16]

The incidence of induced Clindamycin resistance among MRSA was 39%. Similiar results were obtained from other studies also.[15,79,94] A significant difference was demonstrated between the sensitivity of CA MRSA and HAMRSA to ciprofloxacin. (p=0.008). CAMRSA was found to be more sensitive to antibiotics like Amikacin, Erythromycin, Cotrimoxazole, Tetracycline and Chloramphenicol than HAMRSA, but this fact was not statistically significant as was seen in many other studies also.[1,72]

Our study would be incomplete without mentioning about the Coagulase negative Staphylococci. These were previously dismissed as skin contaminants and are now emerging as important potential pathogens with an increase in the number of severely debilitated patients and increased use of implants in hospitals. CONS is one of the leading cause of nosocomial infections, especially in neonates, immunocompromised individuals and patients with prosthetic implants.[9] More than 30 species of CONS are recognized but only a few are commonly incriminated in human infections. Multi-drug resistant strains are also common now. In our study, 41 clinically relevant CONs were isolated and speciated. *Staphylococcus epidermidis* was found to be the predominant species among CONS (58.5%). Several other studies also identified *Staphylococcus epidermidis* as the predominant species.[14,78,95] *Staphylococcus epidermidis* was followed by *Staphylococcus haemolyticus* (14.6%), *Staphylococcus cohini* (10%) *Staphylococcus saprophyticus,*(7%) *Staphylococcus hominis,* (7%) and *Staphylococcus simulans (*2.4%). They were all isolated from patients who were either on intravascular devices or on ventilators.

Staphylococcus haemolyticus was next in our list. They were isolated from babies who were premature/ ventilator assistance and from cutaneous infections. *Staphylococcus saprophyticus* was isolated from urinary tract infections similar to other studies.[47] Antibiotic sensitivity pattern showed 100% sensitivity to vancomycin, linezolid and teicoplanin. MRCONS were less sensitive to Amikacin (69%), Tetracycline (75%) Ciprofloxacin (22%), Cotrimoxazole (20%) Gentamycin and Erythromycin (17%) as compared to MSCONS and the results were similar to other studies.[87,96]

Comparison of phenotypic methods of detection of methicillin resistance with the Gold Standard

Among all phenotypic methods tested for detection of MRSA, Cefoxitin disk diffusion had better sensitivity and specificity as compared to other methods. Both methods of detection, namely, cefoxitin resistance and presence of *mecA* gene by PCR, demonstrated a sensitivity and specificity of 100% respectively. This is in agreement with other studies, [74,80,81,82,83] where as, the sensitivity of oxacillin disk diffusion was 100% and its specificity was only 56%. Similiar results were quoted by Swenson etal in their phase 1 study, but there are many other studies quoting a higher specificity for detection of methicillin resistance by oxacillin disk diffusion testing. [97,98,99,100]

The high false Positivity (45%) of oxacillin disk diffusion could be due to the hyperproduction of beta lactamases and modification of Penicillin binding proteins(PBP), both of which may lead to phenotypic expression of oxacillin resistance .This was corroborated by the fact that all the isolates that were resistant to oxacillin, but sensitive to cefoxitin were negative for *mec A* gene by PCR and their MIC's were between 4-8µg/ml. Propably these strains under antibiotic pressure may evolve in to fully resistant isolates subsequently. These isolates were clinically reported as MSSA, and all the patients infected with these isolates, responded well to antibiotics other than Linezolid, Vancomycin and Teicoplanin. This was an added, indirect confirmation that the isolates were truly methicillin sensitive. Therefore, MIC of oxacillin should be determined for those isolates that show discrepant results between oxacillin and cefoxitin disk diffusion methods. They can then be confirmed by *mecA* gene detection.

Both mannitol salt agar and oxacillin screen agar also showed low specificity for detection of MRSA. This is in accordance with other studies,[92] however there are a few reports of better specificity. [51]

Cefoxitin as disk diffusion method had better sensitivity and specificity. Cefoxitin, a cephamycin, is a potent inducer of the mecA regulatory system than are the penicillins. Several groups of investigators have reported that the results of Cefoxitin disk diffusion method correlates better with the presence of mecA, than the results of disk diffusion using oxacillin.[80, 81,82,83,84,85] Cefoxitin disk diffusion testing is now an accepted method for detection of methicillin resistance by an increasing number of reference laboratories including CLSI. From our study also, we have come to the conclusion that cefoxitin disk diffusion is a better and cheaper method for identification of MRSA. Errors in detection of oxacillin resistance can have serious adverse clinical consequences. False susceptibility results may result in treatment failure and the spread of MRSA, if appropriate infection control measures are not applied. Conversely false resistance may increase health care cost following unnecessary isolation precautions and may lead to over use of glycopeptides and later VRSA might emerge which will make life very difficult for all of us

SUMMARY AND CONCLUSION

- *Staphylococcus aureus* was isolated from 610 out of 5919 positive samples obtained from different infections in the study period .
- Methicillin resistant *Staphylococcus aureus* constituted to 34%(208) out of 610 isolates of *Staphylococcus aureus.*
- Elderly patients were significantly more prone for infections with MRSA as compared to children and young adults.
- In our study, only 8% of infections in neonates were caused by *Staphylococcus aureus* and almost half of these were MRSA and rest (92%) of the infections were caused by Gram negative bacilli as reported in many other studies.
- Most cases of MRSA in our hospital were contributed by surgical site wound infection (25%).
- Methicillin-resistant *Staphylococcus aureus* (MRSA) have also been isolated with increasing frequency from wound and skin infections in patients with diabetes(24%).
- Among all infected burns cases,16% were caused by MRSA.
- In our ICU, 17% of infections were caused by MRSA and the rest were mainly contributed by Pseudomonas aeroginosa,Acinetobacter species and Candida albicans
- The incidence of hospital acquired infections among all MRSAs was about 82% in our study, and the patients were mostly diabetics (24%) or had surgical wound infections(25%).
- The incidence of community based MRSA in our study was about 18% and most cases were contributed by skin infections such as cellulites, folliculitis and abscesses.
- In our study only about 40% of MRSA were sensitive to Amikacin and Tetracycline, and less than 15% were sensitive to Cotrimoxazole, Gentamycin, Erythromycin and Ciprofloxacin. Chloramphenicol alone showed 92% sensitivity as was MSSA too (95%).
- In contrast with other studies, 22% of MSSA isolates were sensitive to Penicillin.
- The incidence of induced Clindamycin resistance (Dtest positive) among MRSA was 39%.
- CAMRSA was found to be more sensitive to antibiotics like Amikacin, Erythromycin, Cotrimoxazole, Tetracycline and Chloramphenicol than HAMRSA, but this fact was not statistically significant.

- In our study, 41 clinically relevant CONs were isolated and speciated. *Staphylococcus epidermidis* was found to be the predominant species among CONS (58.5%). *Staphylococcus epidermidis* was followed by *Staphylococcus haemolyticus* (14.6%), *Staphylococcus cohini* (10%) *Staphylococcus saprophyticus,*(7%) *Staphylococcus hominis,*(7%) and *Staphylococcus simulans (*2.4%).
- Among all phenotypic methods tested for detection of MRSA, Cefoxitin disk diffusion had better sensitivity and specificity compared to other methods.Cefoxitin ,a Cephamycin ,is a potent inducer of mecA regulatory system than are the penicillins.
- All the isolates that were resistant to oxacillin, but sensitive to cefoxitin were negative for *mec A* gene by PCR and their MIC's were between 4-8µg/ml
- Therefore, MIC of oxacillin should be determined for the isolates that show discrepant results between oxacillin and cefoxitin disk diffusion methods. They can then be confirmed by *mecA* gene detection.
- Cefoxitin as disk diffusion method had better sensitivity and specificity. Cefoxitin, a cephamycin, is a potent inducer of the mecA regulatory system than are the penicillins
- Cefoxitin disk diffusion testing is now an accepted method for detection of methicillin resistance by an increasing number of reference laboratories including CLSI.
- We have come to the conclusion that cefoxitin disk diffusion is a better and cheaper method for identification of MRSA and errors in detection of oxacillin resistance can have serious adverse clinical consequences.
- False susceptibility results may result in treatment failure and the spread of MRSA, if appropriate infection control measures are not applied.
- Conversely false resistance may increase health care cost following unnecessary isolation precautions and may lead to over use of glycopeptides and later VRSA might emerge.

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ANNEXURE

1. Blood Agar (BA)

Sterile defibrinated sheep blood	7 ml
Nutrient Agar (melted)	100 ml

Pour about 7 ml of melted Nutrient Agar, as a base, in to sterile Petri dishes and allow setting. This forms a thin base for pouring in the blood agar. Add sterile defibrinated sheep blood (5.7 %) to Nutrient Agar, the latter should be cooled to about 45-50^{o} C before blood is added. Mix well and pour about 15 ml of blood agar over the base in each Petri dish. **Human blood is not recommended for the preparation of blood agar.**

Alternatively blood agar may be made with no agar base.

Use

It serves as an enriched medium and a differential medium for hemolytic organisms. Most common pathogens grow on it.

2 Carbohydrate Fermentation Media

Prepare sugar solutions as described below for different groups of organisms and dispense in 3-4 ml quantities in to test tubes (12 x 100 mm). Introduce Durham's tubes into glucose broth for the detection of gas production. Autoclave at 115^{o} C for 10 minutes. Disaccharides like lactose and sucrose are better filtered and added to sterile basal medium.

3. Decarboxylase Test Medium

Basal medium

Peptone	5.0 gm
Beef extract	5.0 gm
Bromcresol purple	0.01 gm
Cresol red (0.2 %)	2.5 ml
Glucose	0.5 gm
Pyridoxal	5.0 mg
Distilled water	1000 ml

Mix all the ingredients and adjust pH to 6.0.

Amino acids.

L-lysine
L-arginine
L-ornithine

Add to make a final concentration of 1 % each of above.

The basal medium is divided into four equal portions, one of which is distributed in to tubes (1 ml amounts) without the addition of any amino acid. These tubes of basal medium are used as controls. To one of the remaining portions of basal medium add L-lysine to the second portion add L-arginine and to the last add L-ornithine to get final concentrations of one percent each. Distribute these also in one- ml amounts. If DL amino acids are used they should be incorporated in to the media in 2 percent concentration, since the microorganisms apparently are only active against the L forms. Sterilize by autoclaving at 115^{o}C for 10 minutes.

4.**Glucose Phosphate Broth or MR-VP medium**

Dipotassium phosphate	5 .0 gm
Protease peptone	5.0 gm
Glucose	5.0 gm
Distilled water	1000 ml

Suspend ingredients in distilled water and heat slightly to dissolve them Sterilize the tube for 118^{o} C for 15 minutes.

5. MacConkey Agar (MA)

Peptone	2.0 gm
Sodium Chloride	0.5 gm
Bile salt	0.5 gm
Lactose	1.0 gm
Agar	1.5 gm
Distilled water	100 ml

Dissolve the ingredients except lactose in distilled water by heating. Adjust pH to 7.6. Add 1 ml of 1% neutral red solution to every 100 ml of medium with lactose. Sterilize by autoclaving at 121 ^{o} C for 15 minutes

Use
This is a partially selective and a differential medium used for the differentiation of lactose fermenting enteric bacteria.
Note:
MacConkey Agar for exclusive use in faces cultures, may be prepared with bile salts No.3 (Difco) a purified product at a concentration of 0.15 %. Incorporation of this purified bile salt will suppress the growth of enterococci and to some extend commensal coliform bacteria a well.

6. Mannitol Salt Agar

Beef extract	0.1 gm
Protease peptone No.3	1.0 gm
Sodium chloride	7.5 gm
Mannitol	1.0 gm
Agar	1.5 gm
Phenol red	0.0025 gm
Distilled water	100 ml

Mix the ingredients well in distilled water. Sterilize by autoclaving at 121^{o} C for 15 minutes. Pour into sterile Petri dishes.

Use
This medium is recommended for the selective isolation of *Staphylococcus aureus* since most other bacteria are inhibited by high salt concentration. Colonies of *Staphylococcus aureus* are surrounded by a yellow halo indicating mannitol fermentation.

7. Milk Agar

Fresh milk	100 ml
Nutrient Agar containing 3 % agar	200 ml

Boil milk and cool so that the cream layer forms. Remove this completely. This step is repeated two to three times .mix with agar and sterilize by autoclaving at 121 ^{o}c for minutes. Pour as plates or slopes

9.MUELLER-HINTON AGAR (M.H.A)

Beef extract	2.gm
Peptone	7.5gm
Starch	1.5gm
Agar	17.0gm
Distilled water	1000ml

Dissolve the ingredients in one liter of distilled water .Mix thoroughly. Heat with frequent agitation and boil for one minute. Adjust Ph to 7.4+_0.2. Sterilize by autoclaving .Do not over heat

10. Nutrient Agar (NA)

Agar powder	1.5 to 1.8 gm
Nutrient Broth	!00 ml

Mix the agar in nutrient broth and heat to dissolve. When cool adjust the pH to 7.5 – 7.6. Sterilize by autoclaving Pour as plates or slopes. To make deeps, reduce agar concentration to 0.5 %.
Use
This is used as a base for many media. Only non-fastidious organisms will grow on this.

11. UREA AGAR CHRISTENSENS

Urea solutions	
Sodium chloride	5.0 gm
Dextrose	1.0 gm
Tryptcase	1.0 gm
Monopot, phosphate	2.0 gm
Urea	20.0 gm
Distilled water	100 ml
Phenol Red 1% solution (in alcohol)	1.2 ml

UREA AGAR BASE

Agar	1.5 gm
Distilled water	90 ml

Dissolve the ingredients for the solution in distilled water and adjust pH to 6.8. Then add Phenol Red. Sterilize by filtration. Keep this stock solution in the cooler.

Dissolve the agar in distilled water. As sterilized by autoclaving. Cool to 45^{o} C and add 10 ml of urea solution. Dispense in 3-4 ml quantities in 12 x 100 mm test tubes. Allow it to solidify to form a small but and a long slant.

12.CARBOHYDRATE FERMENTATION

Test procedure for aerobes

Inoculate lightly from a young culture from agar slant or broth. Incubate at 37^{o} C for one to five days.

Reading

Positive test is shown by acid production (yellow if bromithymol blue indicator is used and pink if Andrade's indicator is used) and or gas inside the Durham tube.

Eg: E.coli produces acid and gas in glucose; S. typhi acid only.

13.CATALASE TEST

1.Emulsify part of a colony in saline on a clean slide

2. Add a drop of 3 % Hydrogen peroxide.

Reading positive test is indicated by the appearance of "gas bubbles". E.g. Staphylococci

Note:

Growth from a blood agar should not be tested as it might give false positive reaction because of the RBCs.

Include a known positive control always.